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Development and Application of Monoclonal Antibodies to Chikungunya Virus Proteins

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ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne pathogen that causes arthritic disease in humans and is considered a serious health threat in regions where competent mosquito vectors are prevalent. The recent CHIKV epidemic (2004-2014) has resulted in an estimated 1.4-6.5 million cases, involving more than 40 countries. There are no commercially licensed human vaccines available against infection with this re-emerging alphavirus. Treatment of CHIKV rheumatic disease usually involves the use of analgesics and/or non-steroidal anti-inflammatory drugs, with relief often inadequate. Furthermore, the number of monoclonal antibodies (mAbs) specific for CHIKV is limited.

This thesis describes the generation and characterisation of a suite of mAbs that are specific to the E2 or capsid protein (CP) of CHIKV. These mAbs were shown to be reactive in a range of assays including ELISA, Western blot, immunofluorescence and immunohistochemistry. Eleven CP-specific mAbs were capable of recognising isolates that represent the major genotypes of CHIKV, as well as several other alphaviruses. In contrast, five mAbs specific for the E2 glycoprotein were able to distinguish CHIKV from all other alphaviruses tested. Two of these E2-specific mAbs have the ability to provide complete protection against arthritis in a CHIKV mouse model when administered prior to infection. The anti-E2 mAbs have also been used successfully in a sensitive epitope-blocking ELISA to detect anti-CHIKV antibodies in clinical samples. Moreover, we have identified CHIKV in a serum sample from a patient using an antigen-capture ELISA.

The recent re-emergence of CHIKV and its potential threat to human health has called for a better understanding of the virus for improved treatment, prevention and control measures. To do so, we have mapped the binding sites of the anti-CP mAbs with the help of N- and C-terminally truncated versions of the CHIKV CP. Two putative binding regions, residues 1 to 35 and 105-210 of CP, were identified. Competitive binding studies also revealed that five of the CP-specific mAbs recognised a series of overlapping epitopes that are likely to be the nuclear export signal of CHIKV CP. Furthermore, we have confirmed the presence of a smaller, truncated product of CP that may have structural and/or functional significance during viral replication. Evidence was also provided to show that the C-terminus of CP is required for antibody binding.

Several ELISA-based diagnostic tests have been developed for the detection anti-CHIKV antibodies. However, international standardization and validation of these assay are currently limited. The use of human sera collected from infected or uninfected individuals is required for the validation of diagnostic assays, as well as to determine cut-off values. Numerous drawbacks associated with the routine usage of pooled reference sera from CHIKV-infected patients include obtaining sufficient quantities to meet demands, stringent safety procedures and ethical concerns to be taken into account, and the requirement of standardization for each batch of control material. Therefore, a recombinant, chimeric version of a selected anti-CP mAb was created as an alternative positive control in diagnostic kits. The chimeric 5.2H7 mAb was assessed in ELISA and Western blot, and was found to have specificity and sensitivity levels comparable to that of the original mAb. In addition, we have derived a novel method to specifically amplify the variable chain sequences of antibody genes that could be applied towards the generation of any other chimeric mAbs, while avoiding the notoriously troublesome aberrant heavy or light chain genes.

Given the current shortage of widely-available reagents for CHIKV, these specific antibodies present not only useful tools for further investigations on the structure and function of the virus, but may also provide the basis for new diagnostics and treatment of the disease.

DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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PUBLICATIONS DURING CANDIDATURE

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Prow, T. W., Chen, X., Prow, N. A., Fernando, G. J., Tan, C. S., Raphael, A. P., Chang, D., Ruutu, M. P., Jenkins, D. W., Pyke, A., Crichton, M. L., Raphaelli, K., Goh, L. Y., Frazer, I. H., Roberts, M. S., Gardner, J., Khromykh, A. A., Suhrbier, A., Hall, R. A. and Kendall, M. A. (2010). Nanopatch-Targeted Skin Vaccination against West Nile Virus and Chikungunya Virus in Mice. *Small* **6**(16): 1776-1784.

Goh, L. Y., Jones, M. L., Hobson-Peters, J., Nouwens, A., Prow, N. A., Barnard, R. T. and Hall, R. A. (2015). Using mass spectrometry-guided specific primer design for generation of chimeric mouse-human antibodies for Chikungunya diagnostics. *J Immunol Methods*.

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Chikungunya virus, monoclonal antibody, B cell epitopes, capsid protein, glycoproteins, recombinant proteins, chimeric antibody, diagnostic, prophylaxis.

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LIST OF ABBREVIATIONS

5′ RACE	5′ rapid amplification of cDNA ends
$A_{260/280}$	Absorbance 260/280 nm
ABTS	2, 2’-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
Arbovirus	Arthropod-borne virus
BCA	Bicinchoninic acid
BEI	Binary ethylineimine
BFV	Barmah forest virus
C-trunc	C-terminal truncation
C6/36	*Aedes albopictus* mosquito larval tissue cell line
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
C_H	Constant heavy chain
CHIKV	Chikungunya fever
CHIKV	Chikungunya virus
C_L	Constant light chain
COS-7L	*Cercopithecus aethiops* kindney with large T antigen cell line
CP	Capsid protein
CPE	Cytopathic effect
DAB	3, 3-diaminobenzidine
DENV	Dengue virus
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E	Envelope glycoprotein
EB-ELISA	Epitope-blocking enzyme-linked immunosorbent assay
EEEV	Eastern equine encephalitis virus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GSP	Gene-specific primer
HI	Hemagglutination inhibition

HRP	Horseradish peroxidase
HSFM	Hybridoma serum-free medium
IFA	Immunofluorescence assay
IFN	Interferon
IHC	Immunohistochemistry
IRF	Interferon regulatory factors
kDa	Kilo dalton
mAb	Monoclonal antibody
MAC-ELISA	IgM antibody-capture enzyme-linked immunosorbent assay
MIA	Microsphere-based immunofluorescence assay
MOI	Multiplicity of infection
N-trunc	N-terminal truncation
NC	Nucleocapsid core
NES	Nuclear export signal
NLS	Nuclear localisation signal
nsP	Non-structural protein
OD	Optical density
ONNV	O'nyong nyong virus
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
pE2	Precursor E2
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with 0.05% Tween 20
PCR	Polymerase chain reaction
PFA	Para-formaldehyde
PI	Percentage inhibition
PNGaseF	Peptide-N-glycosidase F
PRNT	Plaque-reduction neutralization test
PW	PathWest
QHFSS	Queensland Health Forensic and Scientific Services
QIMR	Queensland Institute of Medical Research
rCap	Recombinant full-length capsid protein

RE	Restriction enzyme
RPMI	Roswell Park Memorial Institute medium
RRV	Ross river virus
RT	Reverse transtriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
sCP	Small, truncated capsid protein
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf21	*Spodoptera frugiperda* 21 ovary cell line
Sf9-ET	*Spodoptera frugiperda* 9 ovary easy titre cell line
SFM	Serum-free medium
SFV	Semliki forest virus
SINV	Sindbis virus
SOE-PCR	Splice-overlap extension polymerase chain reaction
$TCID_{50}$	Tissue culture infectious dose 50%
UTR	Untranslated region
VEEV	Venezuelan equine encephalitis
Vero	African green monkey kidney epithelium cell line
V_H	Variable heavy chain
V_L	Variable light chain
VLP	Virus-like particle
WEEV	Western equine encephalitis
WHO	World health organisation
WNV	West Nile virus

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION TO ALPHAVIRUSES

Alphaviruses are small, enveloped, positive-sense single-stranded RNA viruses that are recognised as the etiological agents of severe polyarthritis/polyarthralgia and encephalitis. These viruses are the cause of some of the worlds' most important emerging infectious diseases and are responsible for significant global health issues. Belonging to the *Togaviridae* family, the genus *Alphavirus* comprises more than 40 recognised members that have been categorised into at least seven antigenic complexes based on serological cross-reactivity against other viruses of the same genus **(Table 1.1)**. Four of these serogroups – represented by Semliki Forest, eastern equine encephalitis, western equine encephalitis and Venezuelan equine encephalitis – encompass most of the medically significant alphaviruses. Alphaviruses cause a variety of human diseases, ranging from mild febrile illness to severe polyarthritis and encephalitis, with the former generally associated with 'Old World' viruses and the latter with 'New World' viruses (i.e. eastern, western and Venezuelan encephalitis viruses) (Powers and Logue, 2007; Suhrbier *et al.*, 2012). These viruses are transmitted by blood-sucking arthropods, typically the mosquito, and can replicate in both arthropod and vertebrate hosts.

The Semliki Forest group consists of Old World viruses that are associated with rheumatic diseases. This includes chikungunya virus (CHIKV), Ross River virus (RRV), o'nyong-nyong virus (ONNV) and Semliki Forest virus (SFV). Co-infections of viruses from this serogroup have been reported, notably with other mosquito-borne viruses such as dengue virus (DENV) (Khai Ming *et al.*, 1974; Mitchell *et al.*, 1987; Kumar *et al.*, 2012).

Table 1.1. Alphavirus species and antigenic relationships (L'Vov D *et al.*, 1979; Olaleye *et al.*, 1988; Weaver *et al.*, 1993; Weaver *et al.*, 1997; Hommel *et al.*, 2000; Powers *et al.*, 2001).

Antigenic complex	Species
Barmah Forest	Barmah Forest virus (BFV)
Eastern equine encephalitis	Eastern equine encephalitis virus (EEEV)
Middelburg	Middelburg virus (MIDV)
Ndumu	Ndumu virus (NDUV)
Semliki Forest	Chikungunya virus (CHIKV)
	Mayaro virus (MAYV)
	O'nyong nyong virus (ONNV)
	Subtype: Igbo-Ora virus
	Ross River virus (RRV)
	Subtype: Bebaru virus (BEBV)
	Subtype: Getah virus (GETV)
	Subtype: Sagiyama virus (SAGV)
	Semliki Forest virus (SFV)
	Subtype: Me Tri virus (MTV)
	Una virus (UNAV)
Venezuelan equine encephalitis	Cabassou virus (CABV)
	Everglades virus (EVEV)
	Mosso das Pedras virus (MDPV)
	Mucambo virus (MUCV)
	Paramana virus (PARAV)
	Pixuna virus (PIXV)
	Rio Negro virus (RNV)
	Tonate virus (TONV)
	Trocara virus (TROCV)
	Subtype: Bijou Bridge virus (BBV)
	Venezuelan equine encephalitis virus (VEEV)
Western equine encephalitis	Aura virus (AURAV)
	Buggy Creek virus (BCRV)
	Babanki virus (BBKV)
	Fort Morgan virus (FMV)
	Highlands J virus (HJV)
	Kyzylagach virus
	Sindbis virus (SINV)
	Ockelbo virus
	Western equine encephalitis virus (WEEV)
	Whataroa virus (WHAV)
Unclassified	Salmon pancreas disease virus (SPDV)
	Sleeping disease virus (SDV)
	Southern elephant seal virus (SESV)

1.2 CHIKUNGUNYA VIRUS

CHIKV was first isolated in 1952 in Tanzania from human serum, *Aedes* spp. and *Culex* spp. mosquitoes (Robinson, 1955; Ross, 1956). However, the first descriptive characterisation (i.e. clinical symptoms) of the disease was recorded by David Bylon in 1779 during an epidemic in Batavia, now known as Jakarta (Carey, 1971). The word chikungunya, derived from African dialect, Makonde, means "that which bends up", referring to the cramped posture resulting from the debilitating arthralgic condition of diseased individuals (Enserink, 2006; Sourisseau *et al.*, 2007).

1.3 EPIDEMIOLOGY, TRANSMISSION & PATHOGENESIS

Since its discovery, CHIKV has been responsible for regular epidemics primarily in Africa, Asia and most recently, Europe and the Americas **(Figure 1.1)**. Sporadic cases were reported in West Africa, from Senegal to Cameroun, and in multiple other African countries where CHIKV is endemic. In the 1960s and 1990s, many epidemics occurred in Asia (Pialoux *et al.*, 2007; Jain *et al.*, 2008). Major epidemics appear and disappear cyclically, usually ranging from 7-20 years (Caglioti *et al.*, 2013). In 2004-2007, the largest epidemic of CHIKV disease was recorded, beginning with an outbreak in Kenya that spread across the Indian Ocean Islands to India, where an estimated 1.4 to 6.5 million cases of infections occurred. During 2005-2006, approximately 255,000 cases of CHIKV disease were reported in the Reunion Island (French Indian Ocean), amounting to an unprecedented 33% of the total population (Ravi, 2006; Schuffenecker *et al.*, 2006; Simon *et al.*, 2006). The virus caused the first autochthonous epidemic outbreak in the north-east of Italy in 2007, with >200 human infections traced back to the same index case (Rezza *et al.*, 2007; Watson, 2007; Angelini *et al.*, 2008). CHIKV has since then been detected in numerous other European countries including France, Germany and the Netherlands (Chretien and Linthicum, 2007; Hassing *et al.*, 2008; Queyriaux *et al.*, 2008; Frank *et al.*, 2011; Grandadam *et al.*, 2011). By 2010-2011, CHIKV infections were being reported in South Asia and New Caledonia in the Pacific Ocean (Ng and Hapuarachchi, 2010; Powers, 2011). Since then, large CHIKV outbreaks were recorded in the Republic of Congo, Cambodia, Indonesia, the Philippines and even the main island of Papua New Guinea (Caglioti *et al.*, 2013).

Due to the increase in international travel, CHIK fever has been identified in nearly 40 countries including Japan and the USA (Schwartz and Albert, 2010; Gibney *et al.*, 2011; Mizuno *et al.*, 2011; Miner *et al.*, 2015), and is listed as a category C priority pathogen by the US National

Institute of Allergy and Infectious Diseases (2011). Between December 2013 and March 2015, the Pan American Health Organisation/World Health Organisation has reported more than 1.2 million suspected and almost 5,000 confirmed cases of indigenously-acquired CHIK fever throughout the Americas involving 44 countries or territories (http://www.cdc.gov/chikungunya/geo/index.html). Furthermore, the Centers for Disease Control and Prevention (CDC) has reported 2,344 cases of infections in 47 states of the USA, as of 10th March 2015 (http://www.cdc.gov/chikungunya/geo/united-states-2015.html). The CHIKV epidemic appears to be continuing with outbreaks recently reported in Australasia and the Caribbean (Horwood *et al.*, 2013; Viennet *et al.*, 2013; Van Bortel *et al.*, 2014).

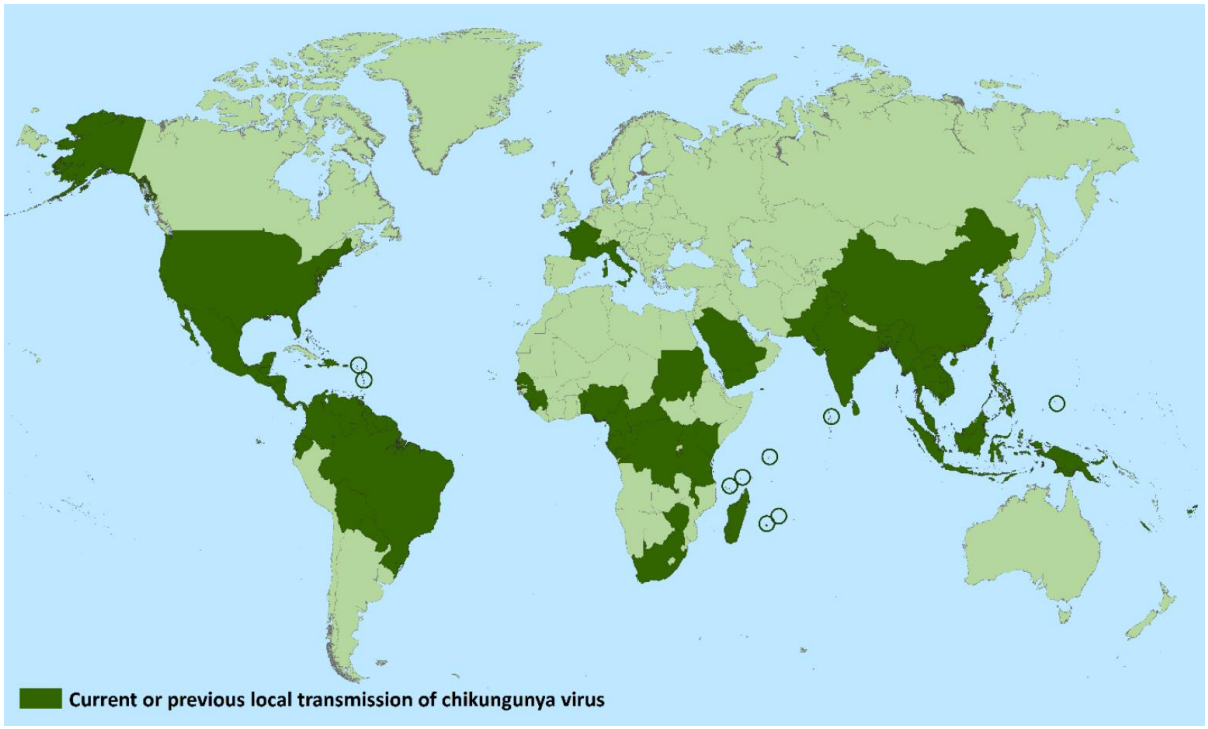

Figure 1.1. Geographical distribution of CHIKV. Data according to the latest update (10th March 2015) of the Centers for Disease Control and Prevention (CDC) website. Map does not include countries of territories where only imported cases have been documented.

CHIKV is believed to be maintained in the wild by sylvatic cycles of transmission between forest-dwelling *Aedes* spp. mosquitoes and non-human primates (Singh and Unni, 2011). Although varying geographically and with ecological conditions, *Ae. furcifer*, *Ae. africanus*, *Ae. neoafricanus*, *Ae. taylori* and *Ae. luteocephalus* are the major species of mosquitoes involved in the maintenance of CHIKV in rural sylvatic cycles (McIntosh and Jupp, 1970; Jupp and McIntosh, 1990; Diallo *et al.*, 1999; Thonnon *et al.*, 1999). Meanwhile, the urban mosquito *Ae. aegypti* has been found to be the most significant vector in urban areas of Africa and in

virtually all of Asia, with humans being the key and most efficient reservoir identified (Powers and Logue, 2007).

Three lineages of CHIKV, namely Asian, West African, and East/Central/South African (ECSA), with distinct genotypic and antigenic characteristics, have been identified (Schuffenecker *et al.*, 2006). The divergence of each distinct lineage reflects, to some extent, the path of global transmission and occasional outbreaks (Caglioti *et al.*, 2013). Phylogenetic analysis showed that the CHIKV strains circulating currently share a common ancestor that existed within the last five centuries (Volk *et al.*, 2010). Despite their proximal geographical distance, the West and ECSA African lineages did not cluster together, indicating limited genetic exchange between the two lineages on the continent. There is, however, evidence that the West African and ECSA lineages may co-circulate in enzootic cycles (Volk *et al.*, 2010). The acquisition of an A226V mutation in the E1 glycoprotein of ECSA CHIKV isolates, as observed in the La Reunion outbreak starting in 2005, has been suggested to be responsible for the increased transmissibility of the virus by the widely-distributed *Ae. albopictus* mosquito (Vazeille *et al.*, 2007). In addition to the lack of herd immunity in the region, the A226V mutation was thought to be a significant contributing factor to the abrupt and escalating nature of the outbreak. This was mainly due an altered vector specificity, allowing the mutated virus to adapt well to replicating in *Ae. albopictus* (Tsetsarkin *et al.*, 2007). The mutated strain spread from the Indian Ocean to East Africa and Asia, and eventually caused the CHIKV outbreak in Italy (Angelini *et al.*, 2007).

Virus strains responsible for the outbreaks in the Caribbean belongs to the Asian genotype, possibly introduced by travellers from Asia where epidemics have been occurring since the 1960s (Lanciotti and Valadere, 2014; Halstead, 2015). While *Ae. aegypti* mosquitoes are widespread in the Caribbean region, *Ae. albopictus* mosquitoes also found in the Americas and on a number of Caribbean islands (Van Bortel *et al.*, 2014). The presence of both *Ae. aegypti* and *Ae. albopictus* mosquitoes, susceptible human amplifying hosts and the ability of CHIKV to transmit in temperate climates has since resulted in comparisons of its potential spread in the US to that of the West Nile virus (WNV) epidemic in the early 2000s (Higgs, 2014). *Ae. albopictus* mosquitoes have also established themselves in most European countries, possibly due to a warmer climate in the past decade, and is known to be highly-active especially during the summer (Chretien and Linthicum, 2007; Queyriaux *et al.*, 2008). Currently, CHIKV's urban transmission cycle relies primarily on *Ae. aegypti* and/or *Ae. albopictus* mosquitoes to initiate human-mosquito-human transmission, utilising humans as reservoirs/amplifying hosts.

The primary mode of CHIKV's urban transmission to vertebrates is through the bite of an infected mosquito. However, in the recent Indian Ocean epidemics, numerous cases of infection were the result of mother-to-child transmission, with a high neonate morbidity rate of up to 100%, and majority (50-90%) presenting with severe illness (Ramful *et al.*, 2007; Gerardin *et al.*, 2008). Following transmission, CHIK replicates in the skin prior to dissemination into the bloodstream, eventually infecting the liver and joints (Talarmin *et al.*, 2007). Infection with CHIKV results in a brief viraemia, usually 5-7 days, followed by an acute onset of disease 2-4 days after infection (Schwartz and Albert, 2010). A wide range of cell types and tissues have been shown to be infected by arthritogenic alphaviruses, including monocytes and/or macrophages, dendritic cells, muscle cells, periosteum and possibly keratinocytes. Widespread infection of these cells and the associated inflammatory immune responses probably account for the acute symptoms caused by CHIKV and other closely-related viruses (Suhrbier *et al.*, 2012, and references therein). The rise in viral titre coincides with disease onset, triggering the activation of an innate immune response, the hallmark of which is represented by the production of type I interferons. Patients typically clear the virus approximately one week post-infection, at which time there is evidence of CHIKV-specific adaptive T cell and antibody-mediated responses. Virus-specific IgM, and sometimes IgG, can be detected as early as 3-4 days after symptom onset (Blacksell *et al.*, 2011; Gibney *et al.*, 2011). An estimated 30-40% of infected adults experience chronic or recurrent arthralgia and/or arthritis (Schwartz and Albert, 2010).

1.4 CLINICAL FEATURES

CHIKV causes a DENV-like illness in humans, characterised by fever, myalgia, a rash which is usually maculopapular in appearance, followed by acute and chronic polyarthralgia/polyarthritis that primarily affects the peripheral small joints (Robinson, 1955; Tesh, 1982). The incapacitating arthralgic syndrome typically resolves within weeks to months, but can be protracted (Borgherini *et al.*, 2008; Hoarau *et al.*, 2010; Suhrbier *et al.*, 2012).

The disease has a sudden onset, with an incubation period between 3-12 days (Mohan, 2006). During the initial viraemic phase, body temperature rises rapidly to around 40 °C and may be accompanied by a rigor. Chikungunya (CHIK) fever corresponds to the period of viraemia and is thought to be related to the ability of the virus to induce large amounts of interferon (IFN) (Carey *et al.*, 1969). Headaches are very common, and non-pruritic maculopapular rashes are often seen appearing on the face, limbs and trunk of the body in 80% of cases, 4-8 days after

the initial illness (Griffin, 2007). The most significant manifestation of CHIKV, allowing a differential diagnosis from DENV, is the excruciating joint pain that occurs with virtually every adult clinical case (Fourie and Morrison, 1979; Economopoulou *et al.*, 2009). The arthralgia is usually symmetrical and peripheral, noted mainly in the fingers, wrists, elbows, knees, ankles and toes. Affected joints exhibit tremendous sensitivity and swelling typically lasting for weeks or months. Paraesthesia of the skin over these joints is common, suggesting neurological involvement (Powers and Logue, 2007). Normally, the acute febrile illness resolves within a few days, whereas the morbidity associated with prolonged joint pain persists typically from weeks to months, thus, resulting in serious economic and social implications for both the infected individuals and their communities. There have also been documented cases of CHIKV-induced arthralgia lasting for several years, with up to 12% of patients developing chronic joint problems (Brighton *et al.*, 1983; McGill, 1995). Symptoms in children described in a 2008 study included fever, rashes, headache, gastrointestinal disorders, and arthralgia and/or diffuse pain, with neurological symptoms (up to 73%) often starting less than 24 hours after the onset of fever, 40% of which led to encephalitis (Robin *et al.*, 2008). Other common manifestations in children include lymphadenopathy, conjunctival injection, swelling of eyelids and pharyngitis (Jadhav *et al.*, 1965). Hemorrhagic fever was also observed in nearly 8% of infected patients in a study involving 639 children in Bangkok (Nimmannitya *et al.*, 1969).

Although CHIKV disease is usually self-limiting, recent epidemics has seen the virus associated with some severe disease manifestations and mortality, primarily amongst elderly patients with co-morbidities, and the very young through mother-to-child transmissions (Robillard *et al.*, 2006; Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010). In the 2005-2006 Reunion Island CHIKV outbreak, babies of 38 mothers who showed symptoms during delivery, or if the new-borns became ill in the first days of life, were found to be infected with CHIKV by detection of viral RNA via RT-PCR and/or presence of specific anti-CHIKV IgM. All neonates were found to be symptomatic and presented with clinical symptoms 3-7 days post-infection (Ramful *et al.*, 2007). In two other studies relating to the same outbreak, vertical transmission was confirmed with nearly half of the infected new-borns presenting neonatal CHIK disease symptoms such as fever, pain, rashes and peripheral edema. About half of the infected neonates developed serious haemorrhage, disseminated intravascular coagulation, and/or cardiac and neurological manifestations; the latter often leading to permanent neurological, visual and/or movement-

based disabilities. Fetal transmission during pregnancy also led to abortion (Lenglet *et al.*, 2006; Gerardin *et al.*, 2008). Three cases of epidermolysis bullosa were also described for the first time following CHIKV infection (Ernould *et al.*, 2008).

Rare symptoms such as lymphopenia, severe dermatological lesions, lethal hepatitis and encephalitis in both adults and new-borns have also been described recently during the Reunion outbreak (Lenglet *et al.*, 2006; Pialoux *et al.*, 2007; Sourisseau *et al.*, 2007). In April 2006, 213 deaths were reported to be directly or indirectly caused by CHIKV during the Reunion outbreak, leading to a case fatality rate of approximately 1 in 1000 (Josseran *et al.*, 2006).

1.5 VACCINES, TREATMENT & PREVENTION

CHIKV infections have been shown to elicit long-lasting immunity in patients, while infection studies with animal models have demonstrated cross-protection against other alphaviruses (Hearn and Rainey, 1963; Nitatpattana *et al.*, 2014; Gasque *et al.*, 2015). There are no licensed human vaccines available for any alphavirus, although vaccines for CHIKV are in development (Akahata *et al.*, 2010; Wang *et al.*, 2011; Brandler *et al.*, 2013; Metz *et al.*, 2013). Furthermore, no curative or prophylactic treatments are available to combat infection with this re-emerging virus, though development of monoclonal antibody therapy are in progress (Pal *et al.*, 2013). Treatment of CHIKV rheumatic disease is symptom-oriented and usually involves the use of salicylate analgesics and/or non-steroidal anti-inflammatory drugs, with relief often inadequate (Suhrbier *et al.*, 2012).

Neutralizing antibodies are believed to be crucial for providing protection, with recent studies illustrating that polyvalent CHIKV-specific antibodies are able to prevent CHIKV infection and disease in mouse models (Couderc *et al.*, 2009; Gardner *et al.*, 2010).

Means of prevention relies on personal protection against mosquito bites and vector control. The use of insect repellent and wearing bite-proof, protective clothing reduces the chances of individuals getting bitten by mosquitoes. However, the most efficient strategy for controlling viral transmission is based on vector control, especially on interventions that aim to reduce the human-vector contact. The three types of interventions that can be implemented are: (i) the increase of population awareness about the risks of arboviral transmission, (ii) the reduction of larval breeding sites by eliminating water-holding containers and/or by using larvicides, and (iii) the control of local adult mosquitoes by spraying insecticides (Doucoure *et al.*, 2014).

1.6 *VIRAL MORPHOLOGY, GENOMIC STRUCTURE & LIFE CYCLE*

The CHIKV genomic RNA encodes four non-structural proteins involved in virus replication and pathogenesis, and five structural proteins. Recent *in vitro* and *in vivo* biochemical and functional studies have allowed additional elucidation of the virus life-cycle and pathogenesis. Furthermore, the efficiency with which many of these viruses replicate, coupled with the broad range of susceptible and permissive hosts, has allowed them to be used as tools in heterologous gene expression and gene therapy delivery vectors. Taken together, recent discoveries within the alphavirus field have provided a broader perspective of the virus lifecycle and may allow for additional control as well as further exploitation of these important viruses.

As with other alphaviruses, the CHIKV virion contains a nucleocapsid consisting of a linear, positive-sense single-stranded RNA genome of approximately 11.7 kb complexed with multiple copies of the capsid protein (CP) and surrounded by a host-derived lipid envelope (Strauss and Strauss, 1994a). The virion is spherical with a diameter of ~70 nm and contains a ~40 nm nucleocapsid particle (Simizu *et al.*, 1984) **(Figure 1.2)**. Supported by a lipid envelope, the viral E1 and E2 glycoproteins are arranged in an icosahedral lattice with T = 4 symmetry. The glycoproteins assemble as heterodimers that are then grouped as trimers to form 80 knobs on the virion surface (Kielian and Rey, 2006). These glycoprotein spikes are involved in cell receptor recognition, and are essential for cell entry via endocytosis and low pH-dependent membrane fusion.

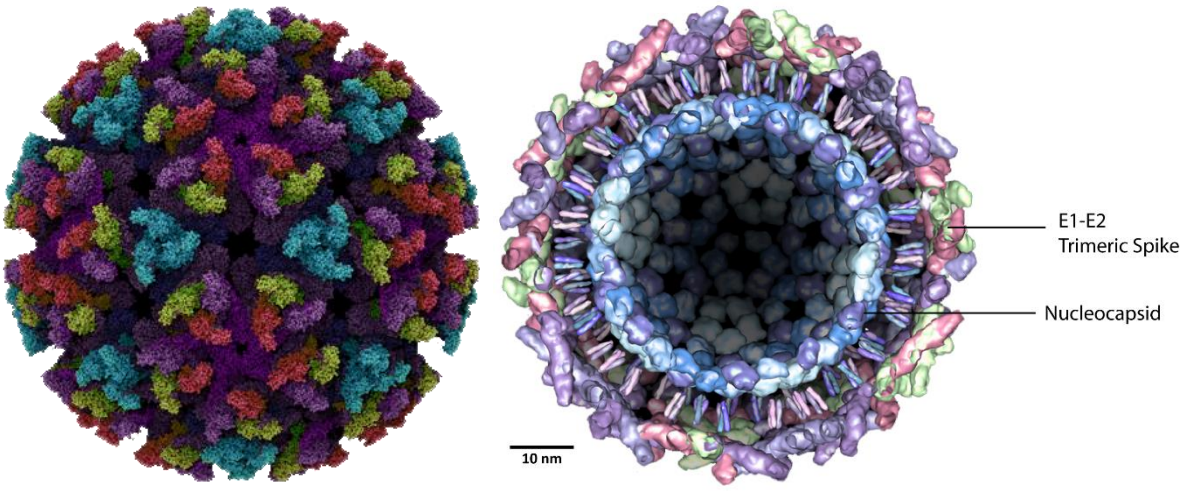

Figure 1.2. Structure of CHIKV. Model of a CHIKV virion (left) generated from information gathered from the Protein Data Bank based on EM density of SINV (PDB ID: 2XFB) and schematic representation of a cross-section of CHIKV particle showing a nucleocapsid core enveloped by a lipid bilayer of trimeric spikes of the E1-E2 glycoproteins.

The CHIKV RNA genome has a 7-methlyguanosine cap at its 5′ end, while the 3′ end is covalently-linked to a poly-A tail **(Figure 1.3)**. Containing two open reading frames (ORFs), the genome encodes for a non-structural polyprotein at its 5′ end and a structural polyprotein at the 3′ end (Strauss and Strauss, 1994a).

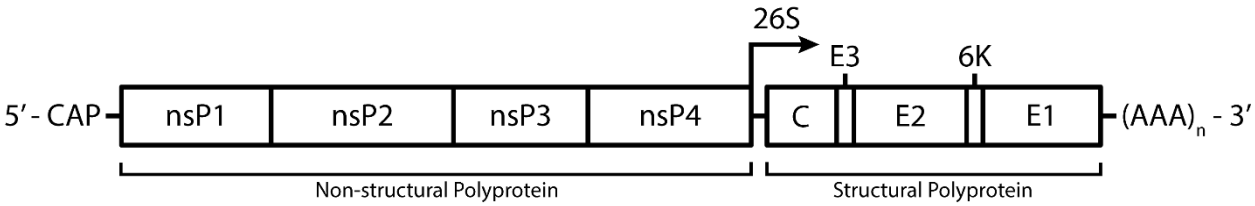

Figure 1.3. Schematic representation of the CHIKV RNA genome. Encoded in the genomic RNA are four non-structural proteins and five structural proteins.

Alphaviruses enter their target host cells by endocytosis in a clathrin-dependent manner via a range of receptors, such as dentritic cell/liver/lymph node-specific ICAM 3-grabbing non-integrins, heparin sulphate, laminin and integrins. However, the precise roles of these receptors have not been established (Griffin, 2007). Upon entry into the cell, the acidic environment of the endosome triggers conformational changes within the viral envelope that causes the E1 fusion peptide to be exposed, mediating virus-host cell membrane fusion **(Figure 1.4)** (Kielian *et al.*, 2010). This enables cytoplasmic delivery of the nucleocapsid core and release of the viral genome (Marsh and Helenius, 2006; Jose *et al.*, 2009).

Non-structural proteins are required for viral replication and are directly translated from the 5′ end of the genomic RNA into two polyproteins - P123 or the larger P1234 (Jose *et al.*, 2009; Sreejith *et al.*, 2012). The latter occurs due to a translational read-through of on opal termination codon at the end of nsP3 (Shin *et al.*, 2012). This is estimated to occur with 10-20% efficiency, as illustrated in Sindbis virus (SINV) (Li and Rice, 1993). Both polyproteins will then undergo processing by the virus-encoded protease located within non-structural protein 2 (nsP2) into their respective intermediate and final component proteins (Strauss *et al.*, 1992). These nsPs assemble to form the viral replication complex, which synthesizes a full-length negative-strand RNA intermediate that serves as a template for the synthesis of both the subgenomic (26S) and genomic (46S) RNAs **(Figure 1.4)**.

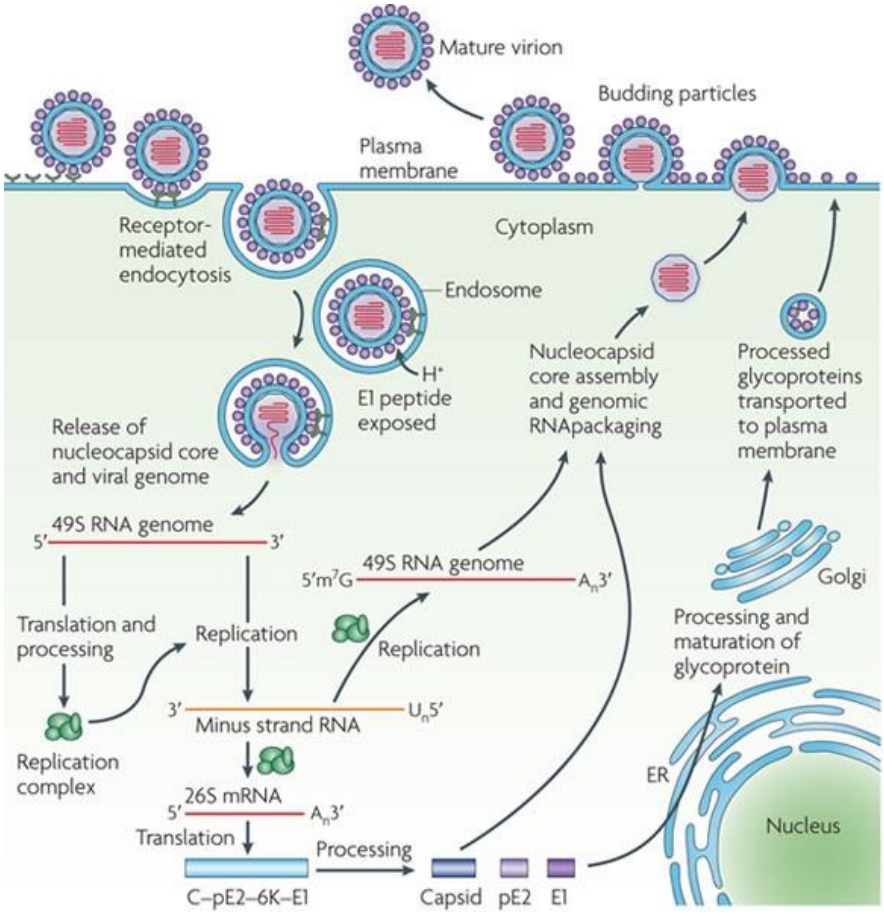

Figure 1.4. CHIKV life cycle, adapted from Schwartz and Albert (2010).

Meanwhile, at the 3′ one-third end of the RNA, downstream of the non-structural protein ORF, a polyprotein precursor (C-pE2-6K-E1) is generated by the translation of a subgenomic 26S mRNA, which is processed by an autoproteolytic serine protease. The capsid protein (CP) is then released from the remaining polyprotein while the pE2 and E1 glycoproteins are generated by undergoing further processing **(Figure 1.4)**. pE2 and E1 proteins associate with one another in the Golgi and are exported to the plasma membrane, where pE2 is cleaved into E2 and E3. Viral assembly is promoted by the binding of viral genomic RNA to CP, and the recruitment of the membrane-associated envelope glycoproteins. The assembled CHIKV particle then buds at the host cell membrane, forming a matured virion (Simizu *et al.*, 1984; Schwartz and Albert, 2010).

1.7 CHIKV NON-STRUCTURAL PROTEINS

Alphaviruses encode four nsPs that are processed from a single non-structural polyprotein. Apart from the formation of the viral replication complex, each nsP has been shown to have its own function. nsP1 is involved in the synthesis of the negative strand viral RNA and has both

guanine-7-methyltransferase and guanyltransferase enzymatic activities (Salonen *et al.*, 2005; Griffin, 2007). nsP2 displays helicase activity required for the unwinding of dsRNA replicative intermediates, harbours an RNA triphosphatase and proteinase for the cleavage of the non-structural polyprotein. It is also known that nsP2 plays a role in shutting-off host cell transcription (Garmashova *et al.*, 2007). nsP3 is part of the replicase unit required for both the minus-strand and subgenomic RNA synthesis, and possesses ADP-ribose 1"-phosphate phosphatase and RNA-binding activity (Jose *et al.*, 2009). Similarly, nsP4 harbours the RNA-dependent RNA polymerase that is also required for RNA replication (Griffin, 2007; Schwartz and Albert, 2010).

1.8 CHIKV STRUCTURAL PROTEINS

The genomic RNA of CHIKV encodes five structural proteins that are processed from a single structural polyprotein. These structural proteins form an integral part in the formation of the virion particle, while possessing specific functions of their own.

1.8.1 Capsid protein

The multifunctional CP plays an important role in the assembly and budding of alphaviruses. The viral genomic RNA is enclosed in a nucleocapsid core (NC) composed of 240 copies of CP in a T = 4 arrangement, leading to an ordered array of projections that are seen as capsomeres on the core (Strauss and Strauss, 1994b). Being the first structural polyprotein synthesized, CP functions as a serine protease to cleave itself from the nascent viral polyprotein precursor (Melancon and Garoff, 1987; Choi *et al.*, 1991). CP then recognises and binds onto the genomic RNA before associating itself with other CP molecules, assembling into an ordered protein to form the NC, encapsidating the viral RNA in the process (Soderlund and Ulmanen, 1977; Ulmanen, 1978; Owen and Kuhn, 1996; Jose *et al.*, 2009). Each CP monomer interacts in a one-to-one ratio with the cytoplasmic domain of the E2 transmembrane spike glycoprotein, thereby driving envelopment and budding of new virions at the plasma membrane of the infected cell (Suomalainen *et al.*, 1992; Zhao *et al.*, 1994; Owen and Kuhn, 1997; Jose *et al.*, 2013). During entry of the virus into a new host cell, each CP on the NC interacts with ribosomes resulting in disassembly of the NC and release of genomic RNA into the cytoplasm (Singh *et al.*, 1997). Apart from its structural significance, studies have also shown that the CP plays a role in the inhibition of host cell protein synthesis (Elgizoli *et al.*, 1989).

The alphavirus CP is organized into three separate regions – I, II and III – each with their distinct functions (Hong *et al.*, 2006). The unconserved N-terminal domain of the alphavirus CP has a large number of positively-charged amino acid residues implicated in non-specific RNA binding (Garmashova *et al.*, 2007). The highly-conserved C-terminal region harbours a globular chemotrypsin-like serine protease and contains the binding site for the cytoplasmic tail of the spike protein (Griffin, 2007). Furthermore, it has been shown that CP contains two nuclear localization signals (NLS) responsible for the karyophilic properties of the protein (Nigg *et al.*, 1991; Favre *et al.*, 1994; Jakob, 1994). Synthetic peptides derived from alphavirus NLS sequences have been used to demonstrate efficient transport of the CP into the nucleolus of both higher and lower eukaryotic target cells (Favre *et al.*, 1994). However, to date, reasons for this import/export of CP to/from the nucleus are still unknown.

1.8.2 Envelope glycoproteins & their signal peptides

The virion envelope of CHIKV consists of a host-derived lipid bilayer in which 240 copies of the E1 and E2 glycoprotein are embedded as 80 trimeric spikes on the virus surface. After the self-cleavage of CP from the structural polyprotein, the remaining envelope cassette is translocated to the endoplasmic reticulum (ER) by signal sequences E3 and 6K (Firth *et al.*, 2008; Snyder and Mukhopadhyay, 2012). E1 and pE2 (precursor to the E3 and E2) are then assembled as heterodimers in the ER. Thereafter, E3 is cleaved from pE2 by furin and glycosylated in the trans-Golgi compartment, and the resultant E1-E2 heterodimers are then transported to the plasma membrane.

The E1 glycoprotein is responsible for fusion of the viral membrane with the endosomal membrane upon entry into host cells. The CHIKV E1 protein has a single N-linked glycosylation site, a short intracytoplasmic tail and a positionally-conserved internal hydrophobic stretch of amino acids in the N-terminal portion that serves as a fusion peptide for virion entry into the cell (Pletnev *et al.*, 2001). The E1 ecto-domain comprises three β-barrel domains – I, II and III. Domain I harbours the amino terminus and is spatially located between the other two domains. The C-terminus of E1 lies within domain III, and the fusion peptide is at the distal end of domain II (Jose *et al.*, 2009).

The E2 protein is involved in host-cell receptor binding and the subsequent clathrin-mediated endocytosis. The E2 glycoprotein is a transmembrane protein that has two N-linked glycosylation sites and contains the most important epitopes for neutralizing antibodies (Davis *et al.*, 1987; Vrati *et al.*, 1988; Metz *et al.*, 2011; Warter *et al.*, 2011). Unlike the E1 protein,

E2 is a thin, long molecule, with a narrow stem that twists around the E1 molecule. The E2 protein also has an exposed leaf-like structure that sits at the top of the spike protein, concealing the distal fusion tip of the E1 glycoprotein domain II (Jose *et al.*, 2009). The carboxy-terminal domain of E2 consists of ~30 amino acids that interact with the NC core (Mukhopadhyay *et al.*, 2006).

The E3 protein serves as a signal sequence for the translocation of the pE2-6K-E1 polyprotein into the ER. An essential step for virus maturation, E3 is then cleaved from E2 by furin in the trans-Golgi complex (Strauss and Strauss, 1994a). The small, 64-amino-acid E3 also mediates proper folding of pE2 and controls the spike functions by interacting with the fusion protein E1. E3 is known to be required for efficient particle assembly, mediating both spike folding and spike activation for viral entry (Snyder and Mukhopadhyay, 2012). The small 6K protein is an acylated protein that is cysteine-rich and hydrophobic in nature. Although only small amounts are incorporated into virions, the 6 kDa protein has numerous functions. It acts as a signalling peptide for E1, is involved in envelope protein processing, membrane permeabilisation, virion assembly, as well as virus budding (Firth *et al.*, 2008).

1.9 CURRENT DIAGNOSTIC METHODS

The advancement in technology and travel has seen mosquito vectors expand to new areas, and diseases spread to previously inaccessible regions. The unprecedented CHIKV epidemic in 2004-2007 underlined the fact that CHIKV is no longer simply a developing world problem. Factors such as increased international travel and the enhanced fitness of *Aedes albopictus* mosquitoes to transmit CHIKV, due to the A226V mutation in the E1 glycoprotein, has seen the virus spread to continents outside of Africa or Asia. Furthermore, in the recent outbreaks, CHIKV infections have been associated with neurological complications, such as encephalitis, which were previously limited only to New World alphaviruses (Rampal *et al.*, 2007; Chandak *et al.*, 2009).

The most suitable test for diagnosing CHIKV is dependent on the phase of disease **(Figure 1.5)**. Acute symptoms generally last for approximately five days, during which viraemia is present and can be confirmed by virus isolation, RT-PCR or antigen detection from the blood of viraemic patients or infected tissues. RNA and antigen from non-viable virus can also be detectable for a few days after this (Sam *et al.*, 2011). CHIKV-specific IgM can be detected 3-8 days after onset of symptoms and can persist for several months (Yap *et al.*, 2010). On the other hand, virus-specific IgG can reach detectable levels 4-10 days after onset of symptoms,

often lasting for years after infection (Kam *et al.*, 2012). Complement fixation and neutralization of viral infectivity are other tests that have been employed to detect CHIKV infection (Olaleye *et al.*, 1989; Warter *et al.*, 2011).

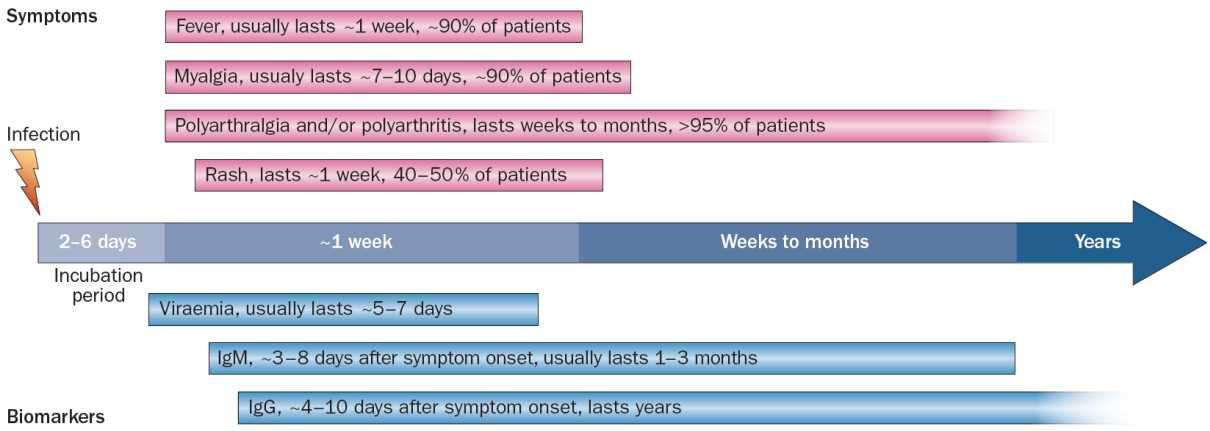

Figure 1.5. Typical course of CHIKV disease in adults (Suhrbier *et al.*, 2012). A schematic representation of the typical course of CHIKV disease, illustrating the time points of typical symptoms (pink) and biomarkers (blue) in boxes. Viraemia is usually followed by fever, myalgia, polyarthralgia and/or polyarthritis. A rash, usually maculopapular in appearance, can appear 2-4 days post-symptom onset. Polyarthralgia and/or polyarthritis and IgG levels in blood can persist for months to years. Virus-specific IgM and IgG can be detected as early as three or four days after symptom onset, respectively.

The gold standards of CHIKV diagnosis are detection of virus or viral RNA. However, virus isolation is time consuming and requires specialised facilities and skills, which are very limited especially in developing countries. Highly-specific and sensitive RT-PCR techniques for CHIKV have been described, but reagents and equipment required to perform these assays are too costly for widespread use (Laurent *et al.*, 2007; Santhosh *et al.*, 2007; Naze *et al.*, 2009; Reddy *et al.*, 2012). Furthermore, detection of RNA is restricted to acute phase samples (day 0-7) **(Figure 1.5)**, and inappropriate storage methods of samples may lead to degradation of virus and RNA.

CHIKV antigen detection can be a good alternative due to the fact that it makes use of simpler, more accessible and established serological platforms such as ELISA, as demonstrated by the diagnosis of DENV using NS1 antigen detection kits (Anderson *et al.*, 2014; da Costa *et al.*, 2014). Although more sensitive results are obtained by gene detection methods such as PCR, viral antigen detection tests are increasingly being used because of their low demand on laboratory equipment and relatively low cost (Grandien, 1996). The use of virus-specific mAbs

in turn provides an increased specificity for disease diagnosis. Antigen detection can be applied to several detection techniques with involving mAbs. The identification of viral proteins in tissue sections via immunohistochemistry and *in vitro* cell or tissue culture-isolated viruses by immunofluorescence are two such examples. Significantly shorter performance times have since been reported with the use of mAbs for the rapid detection of numerous infectious diseases, including dengue, malaria, hepatitis and respiratory viruses (Grandien, 1996; Attallah *et al.*, 2003; Majumdar *et al.*, 2013; Ahmed and Broor, 2014; Maltha *et al.*, 2014).

Unfortunately, there are currently no widely-available CHIKV-specific reagents that can be used in a sensitive antigen detection assay, while in-house CHIKV antigen detection ELISAs described to date do not have their performance characteristics clearly defined (Shukla *et al.*, 2009; Kashyap *et al.*, 2010). In the meantime, serological diagnosis of CHIKV via IgM or IgG capture is more broadly used as it is relatively inexpensive and easier to perform. Nevertheless, the sensitivity of most existing serological diagnostic tests are poorly established, and lack credibility, in terms of specificity, due to the high possibility of false-positive results from cross-reactivity with other closely-related alphaviruses such as RRV, ONNV, Sindbis virus and Barmah Forest virus (Niedrig *et al.*, 2009; Blacksell *et al.*, 2011).

1.9.1 Importance/Principles of a diagnostic assay for CHIKV

Infectious diseases account for approximately a third of deaths worldwide and is the leading cause of death in children below the age of five at almost 70% in 2008 (Black *et al.*, 2010). Numerous common tropical infections, such as dengue, malaria, typhus and leptospirosis, cause non-specific fever similar to chikungunya (Ezzedine *et al.*, 2008; Schilling *et al.*, 2009; Sam *et al.*, 2011; Kumar *et al.*, 2012). Without specific diagnosis, it will be difficult to tell these diseases apart based on clinical symptoms. A study by Baba and colleagues (2013), which included malaria, typhoid, CHIKV, DENV, WNV and yellow fever virus, showed that approximately 40% of patients were infected with a combination of one or more of these pathogens; half of the patients also tested positive with anti-CHIKV antibodies via plaque reduction neutralisation tests. This suggests that the misdiagnosis of arboviral co-infections, combined with the under-reporting and/or lack of disease surveillance can increase the potential for unnoticed and uncontrolled spread of medically-important vector-borne diseases, which may result in underlying public health concerns.

Although no specific vaccines or antivirals are available for CHIKV, there are several advantages for the diagnosis of the infection. It is not uncommon that patients presenting with

undiagnosed fever are empirically and unnecessarily given antibiotics, which is a waste of resources and may contribute to antimicrobial resistance (Sam *et al.*, 2011). Early detection of CHIKV can result in prompt interventions for vector control to contain potential outbreaks. In addition, effective surveillance of the emerging disease, especially in developing countries, can alert the local and global community to impending threats, which is crucial for prevention of large epidemics or further spread.

According to the WHO, the ideal test for any infectious disease should have the following characteristics, labelled "ASSURED": affordable, sensitive, specific, user-friendly, rapid and robust in if different climates, equipment-free, and be delivered to those who need it (Urdea *et al.*, 2006). Furthermore, by combining the detection of both pathogen-specific antigen and serum antibodies, the sensitivity of the test(s) can be greatly increased. The development of a robust, yet reliable point-of-care assay that is rigorously evaluated with well-characterised samples collected from a variety of clinical settings, including infections caused by different CHIKV isolates, will have the potential to be an ideal test. Additionally, if possible, an antigen-detecting kit should be validated for use on mosquitoes, which could greatly benefit vector surveillance programs.

1.9.2 Positive controls for an ELISA-based antibody detection assay

In a diagnostic kit, reference material or positive controls must be included for use with each assay run to confirm the integrity of all test reagents and components. Moreover, to determine if a result is considered positive or negative, the inclusion of a cut-off calibrator at a pre-determined concentration would be required for comparison with sample results. The standardisation and quality of control reagents are integral parts of the optimisation process by which the physical, chemical and biological parameters of an assay are evaluated to ensure that the performance characteristics of the assay are best suited to its intended application. The labour intensive process of optimising controls for an assay is a fundamental and critical step to achieving a reliable and predictable assay performance. These control samples should ideally represent known infected animals (i.e. humans) from the population that will become the target of the assay. Obtaining such reference samples, however, is not always possible (OIE Terrestrial Manual 2014, http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/). Kits used for the detection of serum antibodies against infectious agents commonly utilise human reference sera to establish cut-off values and verify the performance of these tests (Jacobson, 1998). These preparations usually consist of pooled sera

from virus-immune individuals. However, this approach has several disadvantages when used for routine diagnostics. A constant supply of high-titred serum from confirmed cases of CHIKV infections can be challenging to obtain, particularly if CHIKV is rare or exotic in the region. The collection of blood from individuals who are ill, from the young and elderly, or in societies where acquiring blood is forbidden, has caused various human ethical issues as well. Moreover, there are safety concerns of disease contraction, at both the manufacturer and consumer ends, when dealing with potentially infectious human sera. Finally, continuous standardization will be required for each finite batch of material collected due to variable antibody titres and sensitivity or specificity (Hackett *et al.*, 1998). With every varying batch of reference material collected, assays will then be re-calibrated against an international, national or in-house standard prior to aliqouting and storage for routine use (OIE Terrestrial Manual 2014, http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/). Thus, the development of a more consistent, readily available control reagent alternative would help provide serodiagnostic assays with greater reliability and reproducibility in terms of standards.

1.9.3 Chimeric antibodies as alternatives

Chimeric mouse-human mAbs are antibodies that retain their antigen-recognising variable chain regions, but have been engineered to have human constant regions that contain epitopes required for recognition by enzyme-conjugated signal antibodies (Morrison *et al.*, 1984). The creation of humanised chimeric mAbs that bind viral antigen from all genotypes of CHIKV would be a superior alternative to pooled immune human sera as a reference reagent in diagnostic tests. Apart from their capacity to be continuously expressed in stable cell lines, purified chimeric mAbs can offer greater consistency compared to their human-derived serum counterparts. Naturally, with the use of chimeric mAbs as alternative controls, ethical problems regarding the collection of human blood will therefore be no cause for concern. The successful use of chimeric mAbs as positive controls in an ELISA-based diagnostic was first described for the detection of the parasitic protozoan *Toxoplasma gondii* (Hackett *et al.*, 1998). There have since been several other published uses of protein-specific chimeric mAbs as diagnostic control reagents (Schuurman *et al.*, 1997; Ichikawa *et al.*, 1999; Simon *et al.*, 2001; Jones and Barnard, 2007).

1.10 HYBRIDOMA TECHNOLOGY AND THE GENERATION OF MONOCLONAL ANTIBODIES

In 1975, hybridoma technology was invented by Kohler and Milstein (1975). The remarkable and indispensable platform for generating unlimited quantities of pure, mono-specific

antibodies directed against virtually any antigen of interest was seen as a significant milestone in biomedicine and immunology. Monoclonal antibodies (mAbs) are highly-specific and homogeneous species of immunoglobulin molecules produced *in vitro* by a single hybridoma clone generated by the fusion of a myeloma cell with a B lymphocyte from a donor or immunised animal (Zhang, 2012). Hybridoma-derived mAbs have since served as invaluable tools in revolutionising discovery research and therapeutic development in fields such as biology, immunology, oncology and infectious diseases (Little *et al.*, 2000; Weiner, 2007; An, 2010). Furthermore, with the establishment of mAb chimerisation and humanisation, along with the development of transgenic-humanised mice, hybridoma technology has opened up new possibilities for effectively engineering and generating recombinant forms of these mAbs as therapeutics and/or diagnostics. In recent years, mAbs have emerged as the most rapidly expanding class of biopharmaceuticals for a large variety of clinical settings, with 26 out of the currently 28 FDA-approved therapeutic mAbs originating from hybridomas (Reichert, 2011; Zhang, 2012).

MAbs generated from immune hosts by the hybridoma approach often exhibit good binding affinity due to *in vivo* secondary immune responses, and are thus very useful as diagnostic reagents (Nossal, 1992). Detection of viruses via immunoassays with the use of mAbs are generally much faster and/or simpler to perform as compared to other commonly used methods such as virus isolation or detection of viral nucleic acid. Immunoassays also provide greater flexibility and variety (e.g. capture of both viral antigens and/or antibodies) compared to using molecular methods. Examples of mAb-utilising detection techniques include ELISAs, Western blotting, dipsticks, dot blot tests, immunofluorescence assays and immunohistochemistry.

Aside from their use in therapeutics and diagnostics, mAbs are also important research tools to assist investigators in gaining a better understanding of viruses, with emphasis on the structure and function of specific viral proteins. Although alphaviruses have been well-studied, SINV and SFV have been used as the main investigation models for the genus. Given the shortage of widely available reagents for CHIKV, the re-emergence of the virus and its potential threat to human health has intensified the need for new research reagents, and improved diagnostics and treatments. CHIKV-specific mAbs can therefore be tools capable of providing a practical resolution to these demands.

1.11 RESEARCH PROJECT AIMS AND DESIGN

The primary objective of this project was to develop, characterise and evaluate a set of reagents, in the form of mAbs, for chikungunya virus. These mAbs will be useful tools for CHIKV research, diagnostics and potentially disease therapeutics. Additionally, the creation of recombinant CHIKV structural proteins - rE1, rE2 and rCap - will assist in the assessment of mAb production and/or be used as safe diagnostic antigen alternatives. Specific aims of the project were:

i) To generate and characterize a suite of mAbs to CHIKV proteins for use in virus detection and/or immunotherapy;
ii) Express recombinant CHIKV structural proteins to characterise the epitopes recognised by mAbs and assess their use in diagnostic tests;
iii) Genetically engineer and express CHIKV-specific chimeric mouse-human mAbs for use as reference controls in diagnostics; and
iv) Evaluate the use of these mAbs and recombinant antigens for use as diagnostics for CHIKV.

The lack of virus-specific mAbs has resulted in poor serodiagnosis accuracy of CHIKV via commercially diagnostic test kits such as ELISAs and immuno-chromatographic tests (Niedrig *et al.*, 2009; Blacksell *et al.*, 2011). This is mainly due to insufficient affinity of existing antibodies towards CHIKV, as well as the inability to accurately distinguish between CHIKV and other closely-related alphaviruses, resulting in false-positive results from cross-reactivity. It is therefore imperative that CHIKV-specific reagents such as mAbs and antigens, are developed for serodiagnostic assays that boast high sensitivity and specificity. Successful chimerisation of CHIKV-specific mAbs can also be useful as positive controls in place of human reference sera for diagnostic kits. As compared to obtaining potentially infectious human blood, such recombinant alternatives will provide added consistency, supply and safety to existing and future serodiagnostic assays. Furthermore, should the anti-CHIKV mAbs generated in this project provide evidence of protection against disease in animal models before and/or after viral inoculation, they may have potential as therapeutic agents. Finally, these CHIKV-reactive mAbs will undoubtedly be valuable tools for CHIKV and alphavirus research.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. CELL CULTURE

Maintenance of eukaryotic cell or tissue cultures were performed under sterile conditions in a laminar flow cabinet within a physical containment level 2 (PC2) laboratory, unless otherwise stated. Cells were cultured as monolayers, unless otherwise stated, in sterile culture flasks (Greiner Bio-One) of varying sizes in their respective medium containing sterile-filtered and heat inactivated fetal bovine serum (FBS, Gibco, Life Technologies). All cell cultures were supplemented with 50 U penicillin mL^{-1}, 50 μg streptomycin mL^{-1} and 2 mM L-Glutamine (Gibco, Life Technologies), unless otherwise stated. The choice/use of each particular cell line are presented and/or described in detail in their respective material and methods sections of each chapter/paper article.

2.1.1. Mammalian cell lines

COS-7L and Vero cells were propagated in Roswell Park Memorial Institute medium 1640 (RPMI 1640, Lonza) and Dulbecco's Modified Eagle medium (DMEM, Lonza), respectively, supplemented with 2% FBS during maintenance, or 5% FBS for growth. Hybridoma cells were expanded in Hybridoma serum-free medium (HSFM, Gibco, Life Technologies) with 20% FBS, before being weaned off all FBS for harvesting. All mammalian cell lines were incubated in vented-flasks (Greiner Bio-One) at 37 °C with 5% CO_2 in a humidified cell culture incubator (Sanyo Electric). Cells were passaged by removal of supernatant prior to rinsing twice with sterile PBS. Following the wash step, 1 mL of Trypsin-EDTA (Invitrogen, Life Techonologies) was added and cells were allowed to incubate at 37 °C for 5 min. Firm tapping of flasks was performed to detach the monolayer before the cells were thoroughly resuspended in 10 mL of PBS and diluted accordingly, as required, with fresh growth/maintenance media. In the case of hybridoma cells, supernatants were gently removed and stored, leaving behind an appropriate amount for resuspension of cells via gentle agitation. Fresh HSFM was then used to top up to the required volume for further harvesting, or counted (see section 2.1.3.) and expanded in a larger-sized flask.

2.1.2. Insect cell lines

C6/36 (*Aedes albopictus* mosquito) cells were propagated in RPMI 1640 containing 2% FBS or 5% FBS for maintenance or growth, respectively. C6/36 cell lines were incubated at 28 °C

in sealed flasks (Greiner Bio-One) as described above. Passaging of the insect cells are as described previously for mammalian cells, except that Trypsin-PBS (Invitrogen, Life Techonologies) was used instead of Trypsin-EDTA.

2.1.3. Cell counting

When necessary, 10 µL of resuspended cells were diluted 1:1 with trypan-blue dye and loaded onto a haemocytometer with a glass coverslip. The average cell count per chamber grid was doubled and then multiplied by 10^4 to determine the number of cells per millilitre. Cells were then placed back into their respective vessel or appropriately seeded.

2.1.4. Cryopreservation and resuscitation of cell lines

Cell cultures were allowed to expand to logarithmic-phase, typically 70-80% confluency, before being rinsed twice with sterile PBS and trypsinised. In the case of hybridoma cells, cells were simply detached via gentle agitation in their own culture supernatants. Detached cells were then resuspended in fresh media and pelleted for 5-10 min, depending on cell type and amount, at 1,000 rpm on a bench-top centrifuge. Supernatants were removed and the resulting cell pellets were gently resuspended in ice-cold freezing media containing 10% DMSO and 20% FBS in the appropriate growth medium. Cells were then aliquoted into 1 mL cryo-vials (Nunc) and immediately frozen to -80 °C, at approximately 1 °C/min, in a cryo-freeze container (Nalgene).

Cell line resuscitations were initiated by rapidly thawing cells in a 37 °C water bath, followed by the addition of 9 mL of fresh growth media into a 15 mL tube (BD Falcon). Cells were pelleted by centrifugation for 5-10 min at 1,000 rpm. Supernatants were discarded and cells were resuspended in the appropriate amount of fresh growth media. Cells were allowed to expand to at least 60% confluency in their respective flasks, at the appropriate conditions, before replacing with maintenance media containing a lower concentration of FBS.

2.2. STANDARD PROCEDURES INVOLVING THE HANDLING OF VIRUSES

Specific components, including virus isolates, are presented and/or described in detail in their respective material and methods sections of each chapter/paper article. All virus manipulations were performed under sterile conditions in a Class II biosafety cabinet. All CHIKV-related work were executed within a PC3 laboratory, while other viruses were handled in a PC2-approved laboratory.

2.2.1. VIRUS STOCKS

Virus stocks used in this project included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186), CHIKV Asian, Thailand strain ($CHIKV_{THAI}$) (GenBank ID: FJ457921), CHIKV Asian, East Timor strain ($CHIKV_{ET}$) (GenBank ID: GQ433359), CHIKV La Reunion strain ($CHIKV_{IMT}$), RRV T48 strain (RRV_{T48}) (GenBank ID: GQ433359), Sindbis virus MRE16 strain ($SINV_{MRE16}$) (GenBank ID: AF492770), BFV BH2193 strain (BFV_{BH2193}) (GenBank ID: U73745) and Semliki Forest virus (SFV) (GenBank ID: NC_003215). Viral stocks were stored at either -80 °C or in liquid nitrogen, unless otherwise stated. See specific material and methods sections for more detailed information.

2.2.2. Infection of cells

Viruses were allowed to infect cells in serum-free media for 1 h at a multiplicity of infection (M.O.I.) of 0.1, in the appropriately-sized flask (Greiner Bio-One), under the appropriate incubation conditions for the respective cell lines. Following incubation, infectious media were removed and cell monolayers were washed thrice with PBS. Fresh growth media were added and cells were incubated for a further 48-72 h, or until desired cytopathic effects (CPE) were observed, before culture supernatants were clarified by centrifugation at 12,000 x g for 10 min at 4 °C and stored at -80 °C in 0.5 mL aliquots, or used immediately for downstream purposes.

2.2.3. Determination of viral titres using $TCID_{50}$ assay

To perform a 50% tissue culture infectious dose ($TCID_{50}$), Vero cells were seeded into 96-well plates (BD Falcon) at 30-40% confluency and allowed to multiply. Once cells reached logarithmic-phase (70-80% confluency), 50 uL of diluted virus (10^{-3} to 10^{-8}) in complete growth media (DMEM supplemented with 2% FBS and antibiotics) was added to each well, with desired replicates. Plates were then incubated for 3-5 days at 37 °C with 5% CO_2. $TCID_{50}$ was determined by the Reed and Muench method, utilising the number of wells showing CPE.

2.2.4. Determination of viral titres using plaque assay

Virus titres could also be determined through their ability to form plaques on Vero cell monolayers in 6-well plates (BD Falcon). Following an hour of incubation with serially-diluted virus (10^{-3} to 10^{-8}), inoculums were removed and cell monolayers were overlaid with 1% low-melting-point agarose in complete growth media (DMEM supplemented with 2% FBS and antibiotics). Infected cells were allowed to incubate for 3-5 days at 37 °C with 5% CO_2. After

incubation, the agarose overlay was removed prior to the fixing of cells with 10% formalin or 4% para-formaldehyde for 1 hr at room temperature. Monolayers were then stained with 0.5% crystal violet solution for 30 min. Viral titres were obtained by counting the number of plaques per dilution. For greater consistency and accuracy, dilutions exhibiting 20-200 plaques per well were used for calculation.

2.3. STANDARD PROCEDURES INVOLVING THE HANDLING OF DNA

Specific components, including DNA templates, plasmids/constructs, primers and PCR conditions, are presented and/or described in detail in their respective material and methods sections of each chapter/paper article. DNA material were stored at either -20 °C or in a -80 °C freezer, unless otherwise specified.

2.3.1. Quantification of DNA

DNA concentrations were determined by obtaining the ratio of absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) of a sample, using the Nanodrop 1000 UV Spectrophotometer (Thermo Fisher Scientific). A ratio of ~1.8 (± 0.1) is generally accepted as pure DNA.

2.3.2. Polymerase chain reaction

Standard and high-fidelity polymerase chain reaction (PCR) reactions were performed using Taq DNA polymerase (New England Biolabs, NEB) and Phusion DNA Polymerase (NEB), respectively, with compatible buffers provided by the manufacturer. PCR mixes were prepared under sterile conditions in a Class II biosafety cabinet, with all reagents diluted/reconstituted in sterile, nuclease-free or diethylpyrocarbonate-treated water. Deoxynucleotide triphosphate (dNTP) solution mixes (2.5 mM each in water diluted in Tris-HCl to pH 7.0) were obtained from NEB, while primers were custom-made by Integrated DNA Technologies or Sigma-Aldrich. PCR runs were performed using either the Mastercycler EP-S thermo-module (Eppendorf), PCR Express thermal cycler (Thermo Electron Hybaid) or the PCR Sprint thermal cycler (Thermo Electron Hybaid).

2.3.3. DNA Cloning

Directional cloning with restriction enzymes (REs) were performed by incorporating RE sequences into gene-specific primers (GSPs) flanking the genes of interest. DNA fragments were then digested with the appropriate REs and purified using the NucleoSpin Gel and PCR

Clean-up kit (Macherey-Nagel). The receiving vector was digested with the same pair of REs with the inclusion of calf-intestinal alkaline phosphatase (NEB) to avoid self-ligation. Ligation of insert to vector was performed at a 1:3 molar ratio using 3 Weiss units of T4 DNA ligase (NEB) and 1x rapid ligation buffer. Reactions were allowed to incubate at 4 °C overnight prior to bacterial transformation.

For non-directional TA cloning, 3′ A-overhangs were created on PCR products not amplified with Taq DNA polymerase. PCR products were incubated with 0.2 mM of dNTPs, 1U of Taq DNA polymerase and 1x Thermopol buffer (NEB) at 72 °C for 15 min, prior to ligation into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions.

2.3.4. Bacterial transformation

Following ligation, 1-2 µL of the reaction was added to 50 µL DH5α competent cells and incubated on ice for at least 30 min. Cells were then heat shocked for exactly 45 sec at 42 °C and placed immediately on ice for a further 2 min. The cells were recovered by adding 150 uL of sterile SOC media (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubation at 37 °C with shaking for an hour. After recovery, 100 µL of the transformants - and further 10-fold dilutions if required - were plated onto sterile LB-ampicillin plates (15 g/L bacteriological-grade agar in Luria-Bertani medium, supplemented with 20 µg/mL ampicillin) and incubated in a 37 °C incubator for 16-20 hr.

2.3.5. Colony PCR

The colony PCR screening technique was employed to identify successfully transformed bacteria colonies containing the desired ligated plasmid construct(s). Using Taq DNA polymerase (NEB), a standard PCR was performed using post-transformation bacteria samples as templates. Two types of template material could be used for colony PCRs: i) transformed bacteria colonies picked off LB-ampicillin plates using a sterile pipette tip; or ii) culture supernatant of picked colonies that were sub-cultured overnight in LB broth containing ampicillin.

2.4. STANDARD PROCEDURES INVOLVING THE HANDLING OF RNA

Specific components, including RNA templates and primers, are presented and/or described in detail in their respective material and methods sections of each chapter/paper article. All RNA

manipulations, where end product(s) were to be used in further downstream work, were performed under sterile conditions in a Class II biosafety cabinet. Equipment/non-disposable components used for handling RNA were treated with RNase Away (Thermo Scientific), whenever possible.

2.4.1. Quantification of RNA

RNA concentrations were determined by obtaining the ratio of absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) of a sample, using the Nanodrop 1000 UV Spectrophotometer (Thermo Fisher Scientific). A ratio of ~2.0 (± 0.1) is generally accepted as pure RNA. RNA material were stored at -80 °C, unless otherwise specified.

2.4.2. Reverse transcription

Following the extraction of total RNA using TRIzol reagent (Life Technologies) or TRI reagent (Sigma-Aldrich), according to their respective manufacturer's instructions, first-strand synthesis was performed to obtain cDNA for downstream manipulation or PCR. The RNA template was allowed to denature and anneal with the appropriate reverse-GSP in a reaction containing 0.5-2 µg of RNA template, 0.4 µM GSP, 0.4 mM dNTP mix and nuclease-free water to a final volume of 16 µL. Reaction mix was heated to 65-80 °C, depending on annealing temperature of GSPs, for 3-5 min, and placed promptly onto ice. Following that, 1 µL of RNAse inhibitors, 1x reverse transcriptase (RT) buffer (NEB) and 200 U M-MuLV RT (NEB) were added to the initial reaction mix. cDNA synthesis was carried out at 42 °C for 1 hr prior to the inactivation of the RT enzyme at 90 °C for 10 min. cDNA products were stored at -20 °C until required, or used immediately.

2.5. STANDARD PROCEDURES INVOLVING THE HANDLING OF PROTEINS

Specific components and conditions involving protein work are presented and/or described in detail in their respective material and methods sections of each chapter/paper article. Protein material were stored at -20 °C, unless otherwise specified.

2.5.1. Quantification of proteins

A bicinchoninic acid assay (Pierce, Thermo Scientific) was utilised, according to the manufacturer's instructions, to measure total protein concentration at an absorbance of 562 nm.

CHAPTER 3: NEUTRALIZING MONOCLONAL ANTIBODIES TO THE E2 PROTEIN OF CHIKUNGUNYA VIRUS PROTECT AGAINST DISEASE IN A MOUSE MODEL

This chapter has been published as **Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Pyke, A. T., Suhrbier, A. and Hall, R. A.** (2013). Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model. *Clin Immunol* **149**(3): 487-497.

3.1. ABSTRACT

Chikungunya virus (CHIKV) recently caused the largest epidemic ever recorded for this virus involving an estimated 1.4-6.5 million cases, with imported cased reported in over 40 countries. The number of monoclonal antibodies specific for this re-emerging alphavirus is currently limited. Herein we describe the generation and characterization of five monoclonal antibodies specific for the E2 glycoprotein of CHIKV. The antibodies detected a range of CHIKV isolates in several assays including ELISA, Western blot, immunofluorescence assay (IFA) and immunohistochemistry (IHC) without evidence of cross-reactivity with other alphaviruses. Four antibodies also neutralized CHIKV *in vitro*, two of which provided complete protection against arthritis in a CHIKV mouse model when administered prior to infection. Given the current shortage of widely available reagents for CHIKV, these specific antibodies will be useful not only in research, but may also provide the basis for new diagnostics and treatments.

3.2. INTRODUCTION

Chikungunya virus (CHIKV) belongs to a group of mosquito-transmitted arthritogenic alphaviruses that include the Australian Ross River virus (RRV) and Barmah Forest virus (BFV), the African o'nyong-nyong virus, the South American Mayaro virus, and the globally distributed Sindbis viruses (Suhrbier *et al.*, 2012). Alphaviruses are enveloped, single-stranded positive-sense RNA viruses whose ≈11.5 kb genomes encode four non-structural proteins (nsP 1-4) and five structural proteins; capsid, E3, E2, 6K and E1 (Strauss and Strauss, 1994b). CHIKV was first isolated in 1952 in Tanzania and has caused sporadic epidemics of primarily rheumatic disease every 2-50 years mainly in Africa and Asia (Suhrbier *et al.*, 2012). Since 2004, CHIKV has been responsible for a series of unprecedented outbreaks with an estimated

1.4-6.5 million human infections, with imported cases reported in over 40 countries (Suhrbier *et al.*, 2012). Although typically transmitted by *Aedes aegypti* mosquito vectors in urbanized areas, the recent epidemic was associated with the emergence of a new clade of chikungunya viruses, characterized by an amino acid substitution in the CHIKV E1 glycoprotein (A226V), that allowed efficient transmission by *Aedes albopictus* mosquitoes (Kumar *et al.*, 2008). In the past 30 years, *Aedes albopictus* has experienced a dramatic global expansion in its geographic distribution (Lambrechts *et al.*, 2010), and its presence in southern Europe allowed the first autochthonous (endogenously transmitted) CHIKV infections in Italy in 2007 (>200 cases) and in France in 2010 (Suhrbier *et al.*, 2012).

CHIKV disease in humans is characterized by acute and chronic polyarthralgia/polyarthritis, which usually resolves within weeks to months, but can be protracted (Hoarau *et al.*, 2010; Suhrbier *et al.*, 2012). The acute phase of disease is often also associated with an abrupt onset of fever, myalgia, and a rash (usually maculopapular) (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Suhrbier *et al.*, 2012). Although CHIKV disease is usually self-limiting, the recent epidemic has been associated with some severe disease manifestations and mortality, primarily amongst elderly patients with co-morbidities and the very young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010). During the recent epidemic, mother-to-child transmission was also observed and about half the neonates born to viraemic mothers became infected. About half the infected neonates developed serious forms of CHIKV disease characterized by hemorrhage, disseminated intravascular coagulation, and/or cardiac and neurological manifestations; the latter often leading to permanent disabilities (Suhrbier *et al.*, 2012 and refs therein).

Serodiagnosis by IgM and IgG ELISA tests are used as standards for laboratory-based diagnoses of arthritogenic alphavirus diseases, and in-house CHIKV ELISAs have been developed in a number of countries (Suhrbier *et al.*, 2012). Detection of the mosquito-borne virus has also been achieved using PCR-based methods; however, blood samples must be taken during the viraemic period (Edwards *et al.*, 2007; Laurent *et al.*, 2007; Santhosh *et al.*, 2007). Treatment of CHIKV rheumatic disease usually involves the use of analgesics and/or non steroidal anti-inflammatory drugs, with relief often inadequate. There is currently no licensed human vaccine available for any alphavirus, although CHIKV vaccines are in development (e.g. Bharat Biotech) (Akahata *et al.*, 2010). Neutralizing antibodies are believed to be crucial for providing protection, with polyvalent CHIKV-specific antibodies able to prevent CHIKV infection and disease in mouse models (Couderc *et al.*, 2009; Gardner *et al.*, 2010).

CHIKV is a biosafety level 3 pathogen in most countries and is listed as a US National Institute of Allergy and Infectious Diseases category C priority pathogen (Diseases, 2011). The US Army has long recognized CHIKV as a potential biological weapon, and CHIKV is considered a possible agent for bioterrorism (Studies, 2011). Centers for Disease Control and Prevention (CDC), and the Pan-American Health Organization recently completed a preparedness plan for the spread of CHIKV to the Americas due to the fact that all the conditions and vectors exist for such an event (Organization, 2011).

Due to the re-emergence of CHIKV and the potential threat to human health there has been a quest for new research reagents, and improved diagnostics and treatments. Herein we report the generation and characterization of a panel of monoclonal antibodies (mAbs) to the E2 glycoprotein of CHIKV. These antibodies recognized multiple isolates of CHIKV and did not cross-react with other arthritogenic alphaviruses. We illustrate the use of these monoclonal antibodies in Western blot, IFA, IHC, capture ELISA diagnostic assays and as potential prophylactic biological drugs in a mouse model of CHIKV viraemia and arthritic disease.

3.3. MATERIAL AND METHODS

3.3.1. Cell and virus culture

Mosquito cells (C6/36 - *Aedes albopictus*) were propagated in RPMI 1640 supplemented with 2% fetal bovine serum. Cultures were passaged by dissociating the cell monolayer from the flask with trypsin/PBS and were incubated at 28 °C. Vero and COS-7L (African green monkey kidney) cell lines were cultured in DMEM and RPMI 1640, respectively, supplemented with 2% fetal bovine serum. Mammalian cells were passaged by dissociating the surface monolayer from the flask with trypsin/EDTA and were cultured at 37 °C with 5% CO_2. Hybridoma cells were expanded in Hybridoma SFM (Gibco, Life Technologies) with 20% fetal bovine serum at 37 °C with 5% CO_2. All cell cultures were supplemented with 50 U penicillin mL^{-1}, 50 µg streptomycin mL^{-1} and 2 mM L-Glutamine (Gibco, Life Technologies).

Viruses used to infect C6/36 cells for fixed-cell ELISA (Clark et al., 2007) included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186); CHIKV Asian strain ($CHIKV_{ASIAN}$) (GenBank ID: FJ457921); CHIKV Asian, East Timor strain ($CHIKV_{ET}$) (provided by Dr. Alyssa Pyke, Queensland Health Forensic and Scientific Services); RRV T48 strain (RRV_{T48}) (GenBank ID: GQ433359); Semliki Forest virus (SFV) (GenBank ID: NC_003215); Sindbis virus MRE16 strain ($SINV_{MRE16}$) (GenBank ID: AF492770) and BFV BH2193 strain (BFV_{BH2193}) (GenBank ID: U73745). Briefly, cells seeded in 96-well plates were infected at an M.O.I. of 0.1 and incubated for 3-4 days before the culture supernatant was removed and cells were fixed overnight in 20% acetone, 0.2% bovine serum albumin in phosphate-buffered saline (PBS) at 4 °C. Plates were then air-dried and stored at -20 °C until use.

Mock and virus-infected C6/36 cell monolayers were incubated in a similar manner before cells were rinsed in PBS and disrupted by sonication in the presence of BS9 lysis buffer (120 mM NaCl, 50 mM H_3BO_3, 1% Triton X-100 and 0.1% SDS, pH 9.0). The lysate was clarified by centrifugation at 12,000 x g for 10 min at 4 °C and stored at -20 °C (Clark *et al.*, 2007).

3.3.2. Mouse immunization

Groups of three to five female BALB/c mice 6-8 weeks of age were immunized subcutaneously (s.c.) onto the ventral side of the ear using a dry-coated nanopatch as described previously (Prow *et al.*, 2010). Briefly, mice were given 0.5 µg or 5.0 µg of sucrose gradient-purified γ-irradiated $CHIKV_{MAU}$ antigen (strain 06113879 – 2006 Mauritius, provided by Dr. Julian Druce, VIDRL, Australia) with Quil-A saponin (Brenntag Biosector) as adjuvant. An identical

immunization was performed three weeks later with a final boost of 5.0 μg $CHIKV_{MAU}$ antigen given four weeks after the second application. Mice were then challenged with live virus as described previously (Gardner *et al.*, 2010; Prow *et al.*, 2010). Finally, 20 months later, mice were boosted with 10 μg of binary-ethyleneimine inactivated $CHIKV_{ASIAN}$ (Gardner *et al.*, 2010) via subcutaneous injection at the base of the tail four days prior to hybridoma production.

3.3.3. Hybridoma production

Mouse spleens were harvested for hybridoma production by fusion of spleen B cells with MRX63 myeloma cells as previously described (Clark *et al.*, 2007). Hybridomas secreting CHIKV-reactive mAbs were identified by fixed-cell ELISA as described below (section 3.3.4.). Isotype determination of selected monoclonal antibodies was performed using the Mouse Typer isotyping kit (Bio-Rad) according to the manufacturer's instructions.

3.3.4. Fixed-cell ELISA

Fixed-cell ELISAs were performed as described previously (Hall *et al.*, 1988; Clark *et al.*, 2007). Briefly, acetone-fixed plates of infected C6/36 cells were blocked with TENTC blocking buffer (0.05 M Tris-HCl pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.05% (v/v) Tween 20, 0.2% (w/v) casein) for 1 h at 28 °C prior to probing of fixed antigen with hybridoma culture fluid at starting dilution of 1/10, and then serially diluted 2-fold across the plate. After incubation for 1 h at 28 °C, wells were washed four times with PBS with 0.1% Tween-20 (PBS/T) wash buffer and bound antibodies were detected with a HRP-conjugated goat anti-mouse IgG (DAKO) diluted 1:4,000 in blocking buffer. Following a 1 h incubation, the plates were washed six times prior to the addition of 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate solution (0.02% (w/v) ABTS, 0.06% (w/v) H_2O_2 in 200 mM Na_2HPO_4 and 100 mM citric acid solution) for 30 min at 28 °C in the dark. Absorbance was measured at 405 nm using a Labsystems Multiscan EX Type 355 UV plate reader (Pathtec). The criterion for specific recognition of antigen was defined as an OD_{405mn} value of at least 0.25 and at least 2-fold greater than that generated by probing uninfected C6/36 cells with the corresponding antibody dilutions. Control monoclonal antibodies G8, 2F2 and 9E8 specific for RRV, SINV and BFV, respectively, were used in the comparative ELISAs described below (Clancy, 1991; Oliveira *et al.*, 2006).

3.3.5. Recombinant CHIKV glycoprotein expression

CHIKV E2 and E1 constructs were generated by amplifying the respective glycoprotein genes, from cDNA synthesized by reverse-transcription PCR of genomic RNA of $CHIKV_{MAU}$, with primer sets CHIKV E3_E2 Forward 5′-ATATAATAGCTAGCATGAGTCTTGCCATCCCAGTTATG-3′, CHIKV E3_E2 Reverse 5′-TTATAATAGGATCCTGTTCTGATGCAGCATA-3′, CHIKV 6K_E1 Forward 5′-AATTAATTGCTAGCATGGCCACATACCAAGAGG-3′, CHIKV 6K_E1 Reverse 5′-ATATATATGGATCCGTGCCTGCTGAACGACACG-3′, followed by ligation into a pcDNA3.1 (+) vector (Invitrogen) modified to express V5 and histidine tags at the C-terminus of the recombinant proteins. COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were harvested 24 h post-transfection by addition of BS9 lysis buffer and clarified by centrifugation (Hobson-Peters *et al.*, 2008). PNGase F enzyme (Sigma-Aldrich) was used, according to manufacturer's instructions, to cleave N-linked oligosaccharide chains for verification of glycosylation (Setoh *et al.*, 2011).

3.3.6. Immunofluorescence assay (IFA)

Transfected COS-7L cells were fixed onto glass coverslips with 100% ice-cold acetone and incubated with selected mAbs in hybridoma culture fluid for 1 h at 37 °C. In the case of live virus infection, Vero cells were allowed to grow overnight on glass coverslips before being infected with CHIKV at an M.O.I. of 0.1 for 1 h. Cells were then washed twice with PBS and incubated at 37 °C in complete growth medium. At 72 h post-infection, Vero cells were fixed and incubated with anti-CHIKV mAbs at a 1/20 dilution as described above. Coverslips were then washed and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:500 in blocking buffer for 1 h at 37 °C, followed by Hoechst 33342 stain (Invitrogen; 1:1,000 in PBS) for 5 min. Coverslips were mounted with ProLong Gold Anti-Fade reagent (Invitrogen) and imaged using a Zeiss LSM 510 META confocal microscope.

3.3.7. Western blot

CHIKV antigens were prepared as crude, CHIKV-infected cell lysate or transfected COS-7L cell lysates as previously described (Setoh *et al.*, 2011). For reduction and carboxymethylation of antigens, lysates were reconstituted in 0.1 M Tris-HCl and reduced with 1 M dithiothreitol (DTT). Samples were then gently treated with streaming nitrogen for 30 seconds before being

heated at 95 °C for 5 min. Reduced lysates were cooled at 4 °C for 5 min prior to the addition of iodoacetic acid to a final concentration of 1 M. Samples were gassed as described above and incubated at 37 °C in the dark for 1 h (Clark *et al.*, 2007). All CHIKV antigens were prepared in 4 X NuPAGE LDS sample buffer (Invitrogen) and heated at 95 °C for 5 min. The proteins were resolved on 4-12% Bis-Tris precast SDS-PAGE gels (Invitrogen), transferred onto Hybond C nitrocellulose membranes (Amersham) and immune-stained as previously described (Clark *et al.*, 2007). Briefly, membranes were blocked with TENTC blocking buffer for 1 h at room temperature prior to the addition of CHIKV-specific mAbs or anti-CHIKV polyclonal mouse sera diluted 1/20, unless otherwise stated, in blocking buffer. After incubation for another hour, membranes were washed thrice with PBS/T wash buffer and bound antibodies were detected with a HRP-conjugated goat anti-mouse IgG diluted 1:4,000 in blocking buffer. The blots were incubated for a further 1 h before being washed three times with PBS/T wash buffer. Finally, blots were developed in DAB substrate solution (1.5 mM 3,3′-diaminobenzidine, 0.06% (v/v) H_2O_2 in PBS, pH 7.2) for 15 min before reactions were terminated by rinsing with PBS or ddH_2O.

3.3.8. Competitive binding assays

Competitive binding between CHIKV E2 mAbs was assessed as described previously (Hall *et al.*, 2009). Briefly, purified mAbs were biotinylated using the BiotinTag kit (Sigma), according to the manufacturer's instructions. The competitive binding ELISAs were performed in 96 well plates coated with a lysate of $CHIKV_{MAU}$-infected C6/36 cells diluted 1/500 in coating buffer (0.05M sodium carbonate/bicarbonate, pH 9.6). After washing, a pre-defined optimal saturating concentration of each of the unlabelled mAbs (1.3A2 - 1.25 µg/mL; 4.6F5 - 0.078 µg/mL; 4.10C12 - 1.25 µg/mL; 5.2B2 - 20 µg/mL; 5.2H8 - 0.156 µg/mL; and 4G2 - 0.4 µg/mL) was added for 1 h at 28 °C. Without washing, a pre-defined optimal non-saturating dilution of each biotin-labelled 'competitor' mAb (1.3A2 - 1.25 µg/mL; 4.6F5 - 1.25 µg/mL; 4.10C12 – 2.5 µg/mL; 5.2B2 - 20 µg/mL; and 5.2H8 - 5 µg/mL) was added for 1 h at 28 °C. After washing six times with PBS/T, horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen) was added and incubated for 30 min. The wells were washed prior to incubation with ABTS substrate solution.

3.3.9. Antigen-capture ELISA

U-bottom PVC 96-well plates (BD Falcon) were coated with 1 µg of purified anti-E2 mAb at 4 °C overnight in coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6). Purified soluble E2 (sE2) antigen, kindly provided by Stefan Metz (Laboratory of Virology, Wageningen University, The Netherlands), was captured by incubation for 1 h at 28 °C, prior to another hour of blocking as described previously. A second biotinylated anti-E2 mAb that binds a different epitope from the capturing mAb was used as a detecting antibody. After incubation for 1 h at 28 °C, enzyme activity was visualized by the addition of ABTS substrate solution as described previously (Clark *et al.*, 2007). Concentrations of purified sE2 protein were initially determined using a BCA Protein Assay kit (Pierce) by the provider (Stefan Metz, personal communications) and confirmed in our laboratory using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific).

3.3.10. Microneutralization assay

Virus neutralization tests were performed on Vero cells as described previously (Hall *et al.*, 1995) with minor modifications. Antibodies tested were subjected to heat inactivation at 56 °C for 30 min before being diluted with DMEM supplemented with 2% FBS. Polyclonal mouse serum was used at an initial dilution of 1:20, while purified CHIKV-specific mAbs were used at a starting concentration of 5 µg per well. Each well was then examined microscopically for cytopathic effect (CPE) and fixed with 4% paraformaldehyde in PBS for 1 h at room temperature before staining with 0.05% crystal violet for validation of cell viability. Neutralization titers were expressed as the reciprocal of the highest serum dilution where CPE did not occur.

3.3.11. Passive immunization with mAb and virus challenge

Six-week old C57BL/6 mice were subcutaneously inoculated with 400 µg (~15 mg/kg) of monoclonal antibody in 100 µL PBS one day prior to challenge with CHIKV (LR2006-OPY1), and viraemia and foot swelling (arthritis) monitored as described previously (Gardner *et al.*, 2010). Viral titres were calculated as $\log_{10}$ 50% cell culture infectious dose per mL of serum ($\log_{10}$ $CCID_{50}$/ml) using 10-fold serial dilutions on C6/36 cells and detection of infection in individual wells using Vero cell cytopathic effects as described (Rudd *et al.*, 2012). The detection cut off is 2 $\log_{10}$ $CCID_{50}$. Foot swelling over time was determined blinded and as a

group average of the percentage increase in foot height x width (in the metatarsal region) for each foot compared with the same foot on day 0 (Rudd *et al.*, 2012).

3.3.12. Immunohistochemical staining of CHIKV-infected mouse tissue

Tissue samples from control and CHIKV-infected mouse foot pads were fixed in 10% neutral buffered formalin for 24 h at room temperature and decalcified with 15% EDTA in 0.1% phosphate buffer over 10 days before being embedded in paraffin wax. Tissue sections, 5 µm thick, from uninfected and $CHIKV_{REUNION}$-infected mice were collected onto charged slides and deparaffinised with three changes of xylene (2 min each), followed by rehydration through a series of graded ethanol concentrations and finally water. Sections were subjected to antigen retrieval by heating at 95 °C in a citrate-buffer, pH 6 (Target Retrieval Solution, DAKO) for 25 min followed by a 20 min cooling period at room temperature, or by incubation with Proteinase K (DAKO) at room temperature for 10 min. A series of blocking steps were performed at room temperature incubation with (i) Peroxidase Block (DAKO) for 10 min, (ii) 0.15 M glycine in PBS for 15 min, (iii) Antibody Diluent with Background-Reducing Components (DAKO) for 30 min, with a brief rinse in Tris-buffered saline with 0.1% Tween-20 (TBS/T) between each step. The tissue samples were then incubated in 1 µg/mL of purified 4.10C12 mAb at 4 °C overnight, prior to 15 min washing with TBS/T. Antibody binding was visualized using the anti-mouse IgG Envision kit (DAKO) according to the manufacturer's instruction. Sections were counterstained with Meyer's hematoxylin, mounted with Glycergel Mounting Medium (DAKO) and examined under a Nikon Eclipse 51E microscope. Digital micro-photographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and processed with the NIS-Elements F software.

3.3.13. Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 19; SPSS, Chicago, IL, USA). For comparison of two samples, the t-test was used when the difference in the variances was less than four and skewness was greater than minus two and kurtosis was less than two. Otherwise, non-parametric tests were used; the Mann-Whitney U test if the difference in the variances was less than four and the Kolmogorov-Smirnov test if the difference in the variances was greater than 4.

3.4. RESULTS

3.4.1. Production of CHIKV-specific monoclonal antibodies

Five hybridomas secreting antibodies reactive to CHIKV proteins were cloned by limit dilution and mAbs were harvested as clarified culture supernatant. The reactivity of these mAbs (1.3A2, 4.6F5, 4.10C12, 5.2B2 and 5.2H8) towards CHIKV and related alphaviruses were determined by fixed-cell ELISA (Table 3.1). While all five mAbs recognized $CHIKV_{MAU}$, $CHIKV_{ASIAN}$ and $CHIKV_{ET}$ strains by ELISA, none were able to detect proteins from fixed cells infected with the related viruses RRV, SFV, SINV or BFV, suggesting that all mAbs produced were specific to CHIKV. All five mAbs were of the IgG_{2A} isotype.

Table 3.1. Reactivity of CHIKV E2-specific mAbs towards various CHIKV strains and other alphaviruses in ELISA.

Monoclonal Antibody	Reactivity in fixed-cell ELISA						
	CHIKV (Mau)	CHIKV (Asian)	CHIKV (ET)	RRV (T48)	SFV	SINV (MRE16)	BFV (BH2193)
1.3 A2 IgG2a	+++++	++++	+++++	-	-	-	-
4.6 F5 IgG2a	++++	+++++	+++++	-	-	-	-
4.10 C12 IgG2a	+++++	+++++	+++++	-	-	-	-
5.2 B2 IgG2a	++++	+++++	+++++	-	-	-	-
5.2 H8 IgG2a	++++	+++++	+++++	-	-	-	-
G8 IgG2a	+	+	+	+++++	+++++	-	+++
2F2 IgG1	-	-	-	-	-	++++	-
9E8	-	-	N.D.	N.D.	-	-	+++++
2B2 IgG2a	-	-	-	-	-	-	-

The dilution producing the maximum mean absorbance reading was scored as: +++++, OD > 1.0; ++++, OD = 0.75 to 1.0; +++, OD = 0.5 to 0.75; ++, OD = 0.3 to 0.5; +, OD = 0.25 to 0.3.

3.4.2. CHIKV-specific mAbs recognize epitopes on the E2 glycoprotein

To determine the protein specificity of these mAbs, recombinant full-length CHIKV E1 (rE1) and E2 (rE2) glycoproteins were expressed in COS-7L cells. Successful expression of the glycoproteins, along with their C-terminus V5-His epitope tag, was confirmed with the use of anti-V5 mAbs (Figure 3.1).

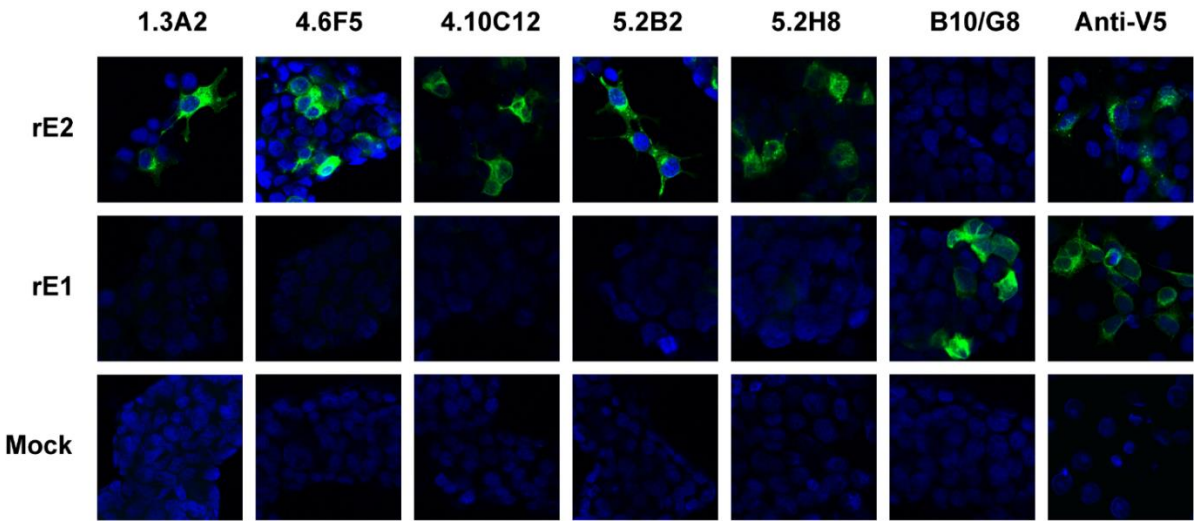

Figure 3.1. Monoclonal antibody reactivity by IFA with acetone-fixed monolayers of COS-7L cells transfected with rE2 and rE1 constructs. Cells were probed with respective mAbs before incubation with an anti-mouse Alexa Fluro 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining. B10/G8 mAbs were generated to the E1 protein of RRV and are cross-reactive with the E1 protein of CHIKV. Successful expression of rE2 and rE1 was demonstrated using anti-V5 mAb. CHIKV mAbs produced in this study reacted with rE2 in IFA, while rE1 was detected using E1-specific cross-reactive RRV mAbs (B10 and G8) produced previously (Broom *et al.*, 1998).

Alphavirus E1 and E2 proteins have asparagine (N) linked glycosylation sites; however, the number of glycosylation sites vary between different species of viruses (Burke and Keegstra, 1979; Rice and Strauss, 1981; Knight *et al.*, 2009). CHIKV-E1 is predicted to be glycosylated at N141, while CHIKV-E2 was expected to have two glycosylation sites at N263 and N273 (Blom *et al.*, 2004). Undigested rE1 and rE2 were shown to be ~51 kDa and ~56 kDa, respectively, while their PNGase F-digested counterparts were ~48 kDa and ~50 kDa in size (Figure 3.2A). This difference in mass is consistent with the single N-linked glycosylation site in E1 and two sites within E2 as elaborated in a previous study (Metz *et al.*, 2011). Further analysis also revealed that the binding of all five anti-E2 mAbs was not dependent on the glycosylation status of the protein as they were capable of detecting the E2 protein in both its original and deglycosylated forms (Figure 3.2B).

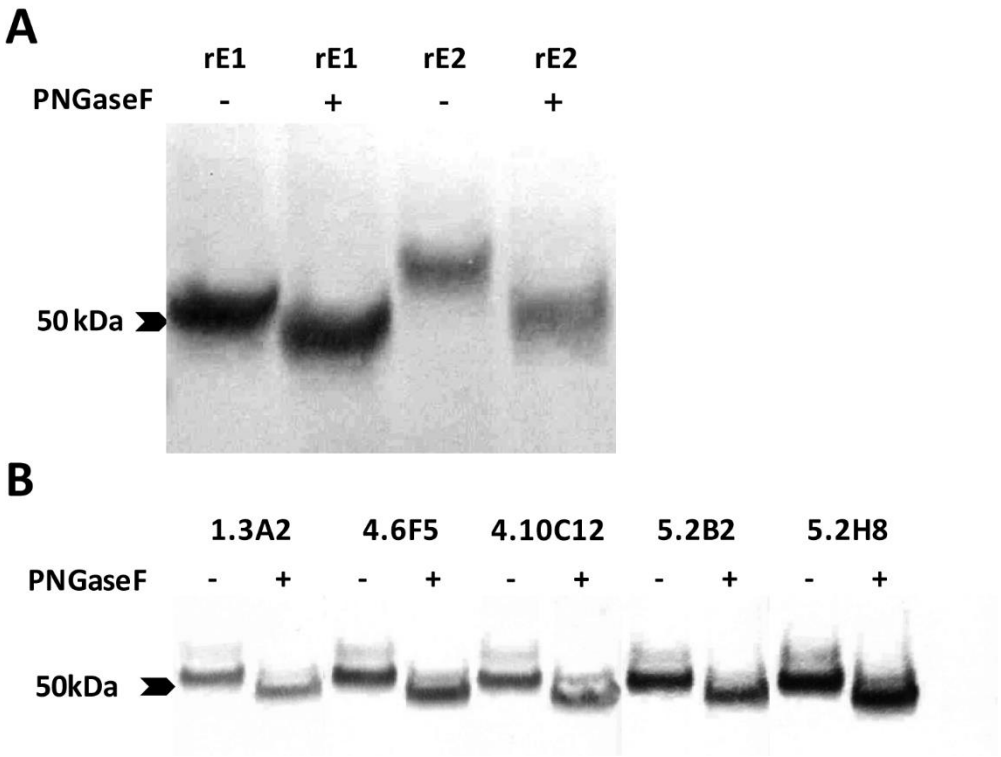

Figure 3.2. Western blot analyses of full-length recombinant E1 and E2 proteins expressed in COS-7L cells with or without PNGaseF treatment. (A) Boiled, unreduced lysates of transfected COS-7L cells expressing rE1 and rE2 were digested with PNGase F (+) or undigested (-); recombinant CHIKV proteins detected using anti-V5 mAb. (B) All five anti-E2 mAbs recognized both glycosylated and unglycosylated forms of E2 under unreduced conditions.

3.4.3. Anti-E2 mAbs recognize reduction-sensitive conformational epitopes

Crude lysates from $CHIKV_{ASIAN}$ infected cells were either reduced with DTT and free sulfhydryl groups carboxymethylated to prevent reformation of disulphide bonds, or were left unreduced prior to SDS-PAGE. Subsequent immunoblotting illustrated that mAbs 1.3A2, 4.6F5, 4.10C12, 5.2B2 and 5.2H8 all recognized rE2 in unreduced samples, but not in DTT-reduced/carboxymethylated samples (Figure 3.3). These results show that the E2-specific mAbs recognized epitopes dependent on intact cysteine residues, suggesting a requirement for secondary structure provided by disulphide bonds for antibody binding.

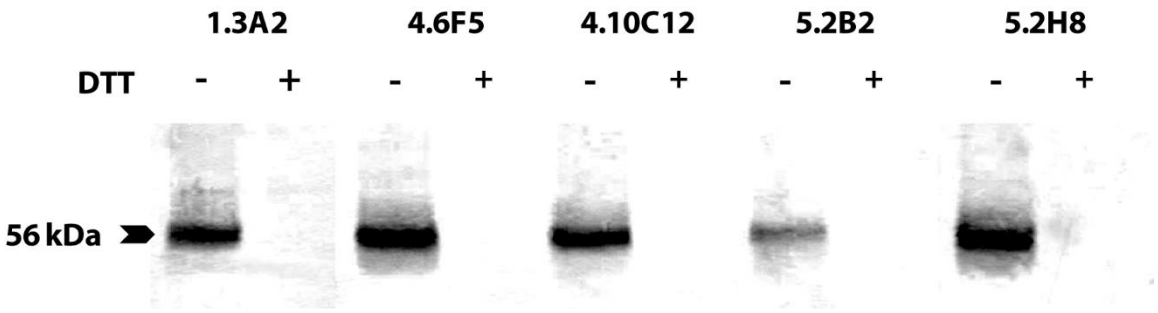

Figure 3.3. Reaction of anti-E2 mAbs to DTT-reduced (+) and unreduced (-) CHIKV-infected cell lysates. DTT breaks disulphide bonds in proteins while the carboxymethylation process caps the free cysteine residues, preventing the reformation of disulphide bonds, and thus, proteins from refolding. All proteins were separated on 4-12% Bis-Tris SDS-PAGE gels.

3.4.4. Competitive binding assay reveals mAbs bind to three different epitopes of E2

To determine the topology of the epitopes recognized by the mAbs on the E2 protein, each antibody was tested in competitive binding assays using ELISA. These assays showed that the five E2-specific mAbs collectively recognize at least three different epitopes on the protein (Figure 3.4). Monoclonal antibodies 1.3A2, 4.6F5 and 4.10C12 inhibited each another, suggesting the epitopes they recognize overlap. Meanwhile, mAbs 5.2B2 and 5.2H8 did not compete with each other, or with any of the other three mAbs, indicating that they bind to different epitopes within E2.

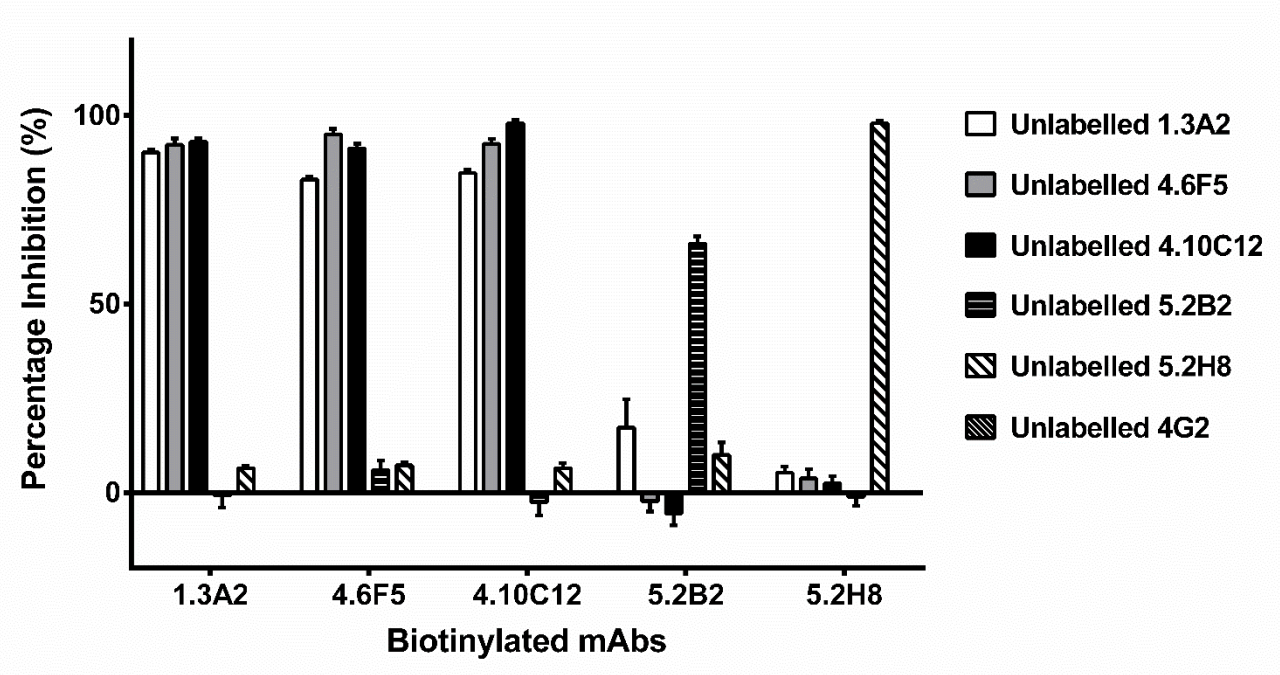

Figure 3.4. Competitive-binding profiles of E2-specific mAbs in ELISA. Antigens in lysates of $CHIKV_{MAU}$-infected Vero cells were adsorbed to 96-well plates at a 1/500 dilution prior to incubation with a saturating dilution of purified, unlabelled anti-E2 mAbs. Without washing, non-saturating dilutions of biotinylated mAbs were then added as 'competitor' antibodies to respective wells. The mean absorbance reading (OD_{405nm}) of four replicates were plotted with bars showing standard error of mean (SEM). 4G2 is a control mAb specific to the E protein of flaviviruses. Assay was optimized to obtain complete inhibition of each biotinylated mAb by its homologous unlabelled competitor.

3.4.5. Use of E2-specific mAbs in antigen-capture assay allows for sensitive detection of CHIKV E2 protein

A double-antibody sandwich antigen-capture ELISA was performed to determine the ability of the anti-E2 mAbs to detect viral proteins in samples. After a series of empirical trials to determine the optimal antibody pairing, two mAbs that bound to different locations of the E2 protein were selected for this assay – purified, unlabelled mAb 1.3A2 was utilized to capture the target antigen, sE2 protein, while a second biotinylated anti-E2 mAb, 5.2H8, was applied as the detecting antibody. Figure 3.5 shows that this assay is capable of detecting the sE2 protein in the picogram range.

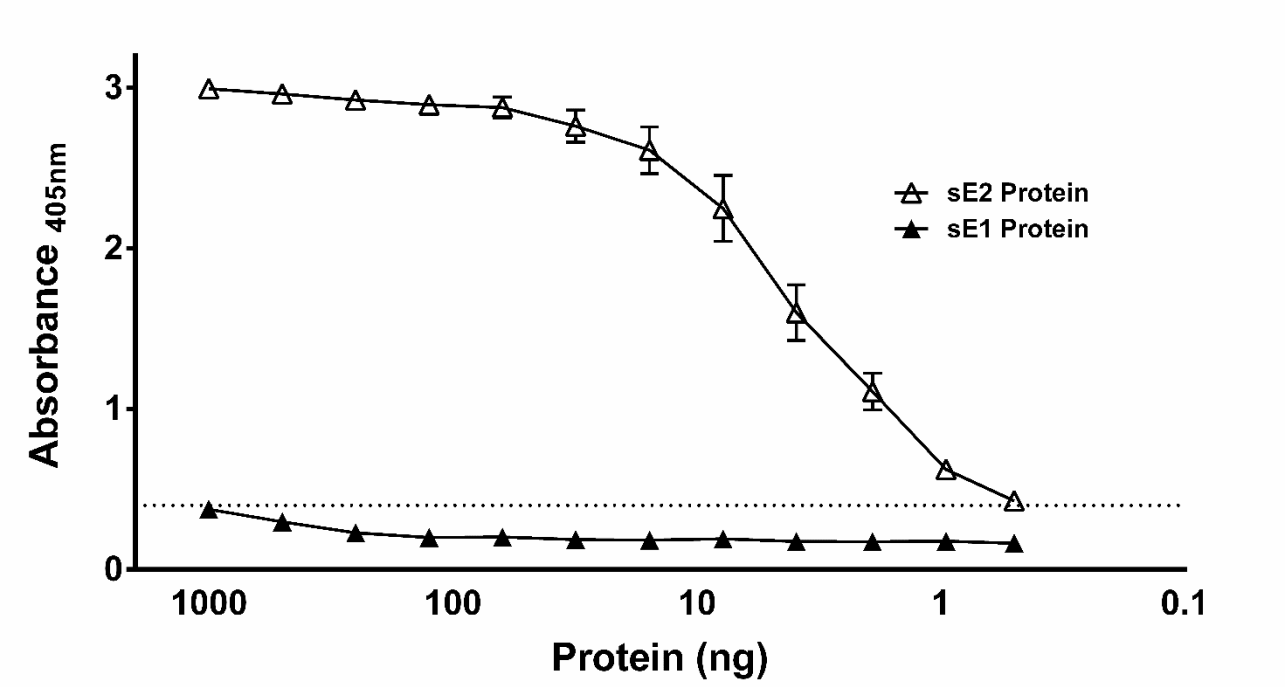

Figure 3.5. Detection of purified soluble E2 protein by anti-E2 mAb in an antigen-capture ELISA. Unlabelled mAb 1.3A2 was coated onto 96-well plates at 1 μg/well prior to incubation with target sE2 protein. The detecting antibody, biotinylated 5.2H8 mAb, was used at 400 ng per well. The mean absorbance reading (OD_{405nm}) of eight replicates from two independent experiments were plotted with bars showing SEM. Dotted line represents the cut-off point, for a positive result, derived from the no antigen control plate (mean OD_{405nm} reading x 2), while sE1 protein (n = 2) was utilized as a negative control for the assay.

3.4.6. Anti-E2 mAbs exhibit virus neutralization in vitro

Virus neutralization assays were undertaken to determine whether the CHIKV E2-specific mAbs were able to neutralize virus *in vitro*. Micro-neutralization tests were performed on Vero cells and revealed that mAbs 1.3A2, 4.6F5 and 4.10C12 were able to efficiently neutralize different CHIKV isolates (Table 3.2), with $CHIKV_{MAU}$ and $CHIKV_{ASIAN}$ representing the East/South African and Asian phylogroups, respectively (Schuffenecker *et al.*, 2006). Monoclonal antibody 5.2B2 showed some neutralization of $CHIKV_{ASIAN}$, but not with $CHIKV_{MAU}$, while mAb 5.2H8 failed to neutralize either CHIKV isolate even at the highest antibody concentration.

Table 3.2. Neutralization activity of E2 mAbs against two CHIKV strains.

	Amount of antibody (µg) required for 80% virus neutralization[a]	
	CHIKV $_{MAURITIUS}$	CHIKV $_{ASIAN}$
Antibody	**100 infectious units**	**100 infectious units**
CHIKV 1.3A2	2.5	2.5
CHIKV 4.6F5	2.5	2.5
CHIKV 4.10C12	2.5	2.5
CHIKV 5.2B2	>5	5
CHIKV 5.2H8	>5	>5
Mouse Sera (CHIKV$_{ASIAN}$) [b]	1/100	1/100
Isotype Control (2B2)[c]	>5	>5

[a] Determined microscopically as 80% reduction in CPE in four replicate wells per antibody dilution.

[b] Dilution factor used instead of antibody concentration.

[c] 2B2 is an IgG2A mAb that reacts to E protein of WNV.

3.4.7. MAbs 1.3A2 and 4.6F5 protect against viraemia and arthritis

A recent study described the use of an adult wild-type mouse model of CHIKV infection and disease, which mimics both the viraemia and rheumatic symptoms seen in humans (Gardner *et al.*, 2010). This mouse model has, for instance, been used to test the efficacy of a number of CHIKV vaccines (Prow *et al.*, 2010; Wang *et al.*, 2011). The model was used to determine whether neutralizing mAbs produced to CHIKV E2 in this study could provide prophylactic protection from viraemia and disease after live CHIKV challenge. Based on their efficient neutralization of CHIKV strains *in vitro*, and the higher yields of purified monoclonal antibodies from their hybridoma cell lines, mAbs 1.3A2 and 4.6F5 were chosen for the *in vivo* study. Mice were inoculated with 400 µg of purified 1.3A2 or 4.6F5 mAb one day prior to challenge with CHIKV. Mice that received these antibodies showed no signs of arthritis as measured by foot swelling (Figure 3.6A) and showed a mean 7-8 log reduction in viraemia when compared with controls (Figure 3.6B). In contrast, control mice (that received PBS or a non-alphavirus reactive mAb) developed clear signs of arthritis that peaked day 6-7 post infection (Figure 3.6A), demonstrating the expected (Gardner *et al.*, 2010) viraemia peaking at 7-8 $\log_{10}$ $CCID_{50}$ on day 2 (Figure 3.6B). These results show that mAbs 1.3A2 and 4.6F5 can provide complete protection against CHIKV arthritis and significantly suppress viraemia when administered prior to infection.

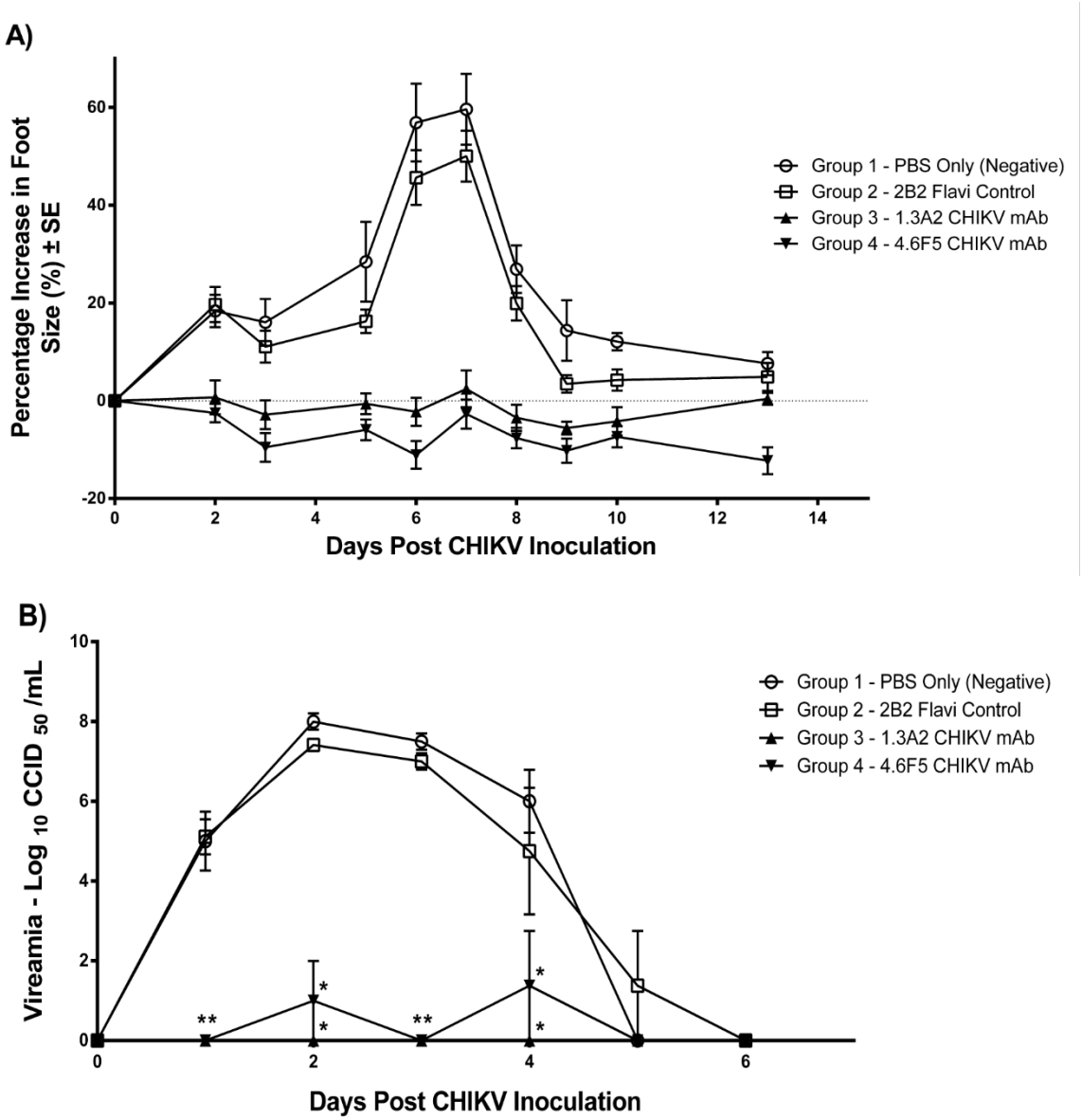

Figure 3.6. Foot swelling and virus replication in mice inoculated with CHIKV-specific mAbs. (A) Arthritis after CHIKV infection. Mice were given 400 µg of mAbs 1.3A2, 4.6F5, 2B2 (anti-flavivirus isotype control) or PBS subcutaneously, one day prior to infection with CHIKV. The data is presented as a group average of the percentage increase in foot height x width for each foot compared with the same foot on day 0 (n = 8 feet per group). PBS and 2B2 groups were significantly different from 1.3A2 and 4.6F5 groups on days 2-10; $p \leq 0.034$, t-tests and Kolmogorov-Smirnov tests. (B) Serum viraemia. The same mice as in A were assessed for viraemia in serum collected at the indicated times (n = 4 mice per group). * indicates $p \leq 0.037$, Kolmogorov-Smirnov tests.

3.4.8. Detection of CHIKV antigens in tissue of infected mice by IHC

To assess the use of the E2-specific mAbs to detect CHIKV in tissues samples, IHC was performed on formalin-fixed, paraffin-embedded samples previously prepared from foot pads of IRF3/7$^{-/-}$ mice infected with CHIKV$_{\text{REUNION}}$ (Rudd *et al.*, 2012). Although staining of infected cells was observed with the use of all anti-CHIKV E2 mAbs when fixed with acetone,

4.10C12 was the only mAb that gave clear reactivity with formalin-fixed tissue sections (Figure 3.7). The detection of CHIKV within keratinocytes in infected mice tissue shown here is consistent with previous studies (Rudd *et al.*, 2012). These results indicate that the mAbs produced in this study can be applied to IHC to specifically recognize CHIKV antigens in formalin-fixed tissue sections.

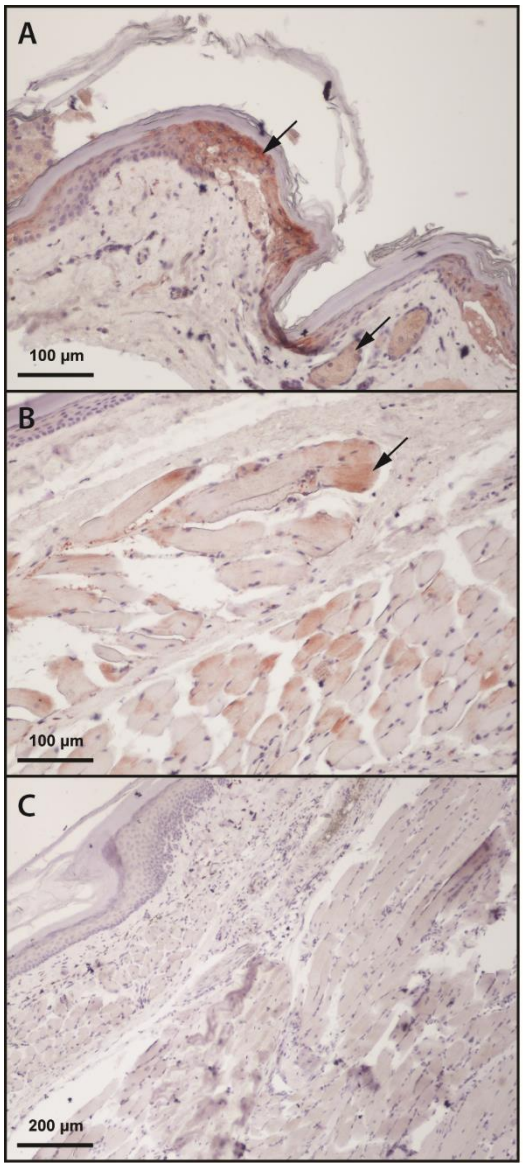

Figure 3.7. Immunohistochemical staining for CHIKV using E2-specific mAb 4.10C12. Five micron sections of formalin-fixed paraffin-embedded uninfected or CHIKV-infected feet tissue of IRF3/7$^{-/-}$ mice were stained using 4.10C12. One microgram of purified 4.10C12 was incubated with tissue sections overnight at 4 °C. Antibody binding was visualized using the anti-mouse IgG Envision kit, DAKO. Staining was observed in (A) epidermal keratinocytes (top arrow) and sweat glands (bottom arrow), and (B) skeletal striated muscle cells, while no staining was observed in the uninfected control (C); A and B were photographed at a magnification of 20x, while the uninfected control C is shown at 10x zoom.

3.5. DISCUSSION

The persistent spread of CHIKV into new locations along with its high epidemic potential poses a serious global threat to areas where competent *Aedes* mosquito vectors are present. The social and economic impact of CHIKV epidemics underlines the demand for accurate diagnostics and effective anti-viral therapeutics, since vaccines are not yet available. This study describes the generation and characterization of five mAbs specific to the E2 glycoprotein of CHIKV. Their specific recognition of three strains of CHIKV (i.e. $CHIKV_{MAU}$, $CHIKV_{ASIAN}$ and $CHIKV_{ET}$) that represent the major global lineages of the virus (Schuffenecker *et al.*, 2006), along with the lack of cross-reaction with other alphaviruses species examined, indicate these antibodies will be useful as specific research and diagnostic tools.

The alphavirus E2 protein is thought to be involved in virus attachment to host cell receptors and contains critical binding sites for neutralizing antibodies as shown in SINV models (Davis *et al.*, 1987; Strauss *et al.*, 1991; Kielian *et al.*, 2010). This is consistent with *in vitro* virus neutralizing activity of mAbs 1.3A2, 4.6F5 and to a lesser extent 4.10C12. These mAbs potentially bind to either the receptor-binding site of the CHIKV E2 protein or an epitope capable of interfering with virus attachment. When we further assessed the ability of mAbs 1.3A2 and 4.6F5 to provide *in vivo* protection in a previously established arthritis mouse model of CHIKV infection (Gardner *et al.*, 2010), a single dose of either mAb gave complete protection against disease progression in all test animals (n = 8). Foot swelling was absent in mice inoculated with the anti-CHIKV mAbs, indicating that the virus had a significant reduction in its ability to proliferate in its primary site of injection. Moreover, the absence of viraemia indicated that the virus introduced was most likely neutralized and was less capable of spreading. The levels of *in vivo* protection provided by mAbs in this study were similar to that recently reported for human anti-CHIKV mAbs by Warter *et al.* (Warter *et al.*, 2011; Fric *et al.*, 2012). When inoculated into AGR.129 (IFN-$\alpha/\beta/\gamma R^{-/-/-}$ and RAG-2^{-}) mice, they showed that at least 250 µg of mAb was required to achieve 80-100% protection from a lethal dose of CHIKV (Fric *et al.*, 2012), as compared to complete protection obtained with 400 µg of mAb in the wild-type mouse model of CHIKV disease used in the current study. Although the practicality of utilizing such antibodies for therapeutic purposes is often questioned, these neutralizing antibodies might be useful to prevent mother-to-child infections (Suhrbier *et al.*, 2012). These results demonstrate the potential of these anti-E2 mAbs to be used prophylactically during an outbreak and warrant future investigation.

Our competitive binding studies indicated that the mAbs produced in this study recognized at least three spatially separated epitopes on E2. These data assisted in the design of a capture assay for CHIKV detection. By selecting two mAbs that bound to different epitopes on the E2 protein, an antigen-capture ELISA was developed for the sensitive detection of CHIKV antigen. Using a purified recombinant CHIKV E2 protein as a standard, this assay was capable of detecting the antigen at picogram levels, demonstrating similar sensitivity to capture assays to detect West Nile virus (WNV) antigen in mosquitoes (Hunt *et al.*, 2002). Further validation of the sensitivity and specificity of this assay is now required using clinical samples to assess its application as a diagnostic assay for the identification of CHIKV in acute infections. These mAbs could also be incorporated into an antigen-capture dip stick format for the detection of CHIKV in mosquito samples, as previously described for WNV (Nasci *et al.*, 2003). Our demonstration that CHIKV E2 antigens can also be detected in infected mouse tissue samples by mAb 4.10C12 in IHC identifies a further application for these reagents as specific tools for the study of CHIKV pathogenesis.

3.6. CONCLUSIONS

In summary, we have produced a panel of mAbs capable of distinguishing CHIKV from other common alphaviruses that cause similar disease syndromes in Australia, representing potentially useful reagents to specifically detect CHIKV in mosquito vectors and/or acute patient samples. However, further studies are required to evaluate their specificity and sensitivity in various diagnostic formats. Successful employment of these antibodies, especially 4.10C12, to detect CHIKV antigen in tissues by IHC further reinforces their potential in a wide variety of diagnostic assays for clinical or research purposes. The evaluation of these anti-CHIKV mAbs against additional medically significant alphaviruses will also allow them to be utilized as diagnostic tools in other regions of the world. Furthermore, we have demonstrated that these E2-specific mAbs neutralize virus both *in vitro* and *in vivo*, highlighting their potential as prophylactic agents against CHIKV infection when genetically engineered into humanized forms. These mAbs will also be useful for studying the structure and function of E2 in viral assembly and virus-host interactions (Akahata and Nabel, 2012). Finally, Metz and colleagues have shown that the CHIKV E2 protein can be a good vaccine candidate and the mAbs developed in this study could prove extremely valuable in terms of monitoring the antigenic authenticity and production of such a vaccine for the disease (Metz *et al.*, 2011).

3.7. ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Goben Pijlman and Stefan Metz for their provision of the purified sE1 and sE2 proteins, Dr. Sonja Hall-Mendalin for the generation of CHIKV-fixed plates for the initial screening of monoclonal antibody production and manuscript review, Jane Wilson for providing CHIKV-infected tissue samples for the IHC, A/Prof. Graham Burgess for the supplement of anti-BFV and SINV mAbs, and Dr. Yin Xiang Setoh for his technical assistance with the recombinant protein expression. Lucas Yuan Hao Goh was supported by the University of Queensland international research tuition award (UQIRTA) from the UQ Graduate School, The University of Queensland, and the ANZ Trustees scholarship for medical research, Queensland.

CHAPTER 4: MONOCLONAL ANTIBODIES SPECIFIC FOR THE CAPSID PROTEIN OF CHIKUNGUNYA VIRUS SUITABLE FOR MULTIPLE APPLICATIONS

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4.1. SUMMARY

Chikungunya virus (CHIKV) is a mosquito-borne pathogen responsible for epidemics of debilitating arthritic disease. The recent outbreak (2004-2014) resulted in an estimated 1.4-6.5 million cases, with imported cases reported in nearly 40 countries. The development of CHIKV-specific diagnostics and research tools is thus highly desirable. Herein we describe the generation and characterization of the first monoclonal antibodies (mAbs) specific for the capsid protein (CP) of CHIKV. The antibodies recognized isolates representing the major genotypes of CHIKV, as well as several other alphaviruses, and were reactive in a range of assays including ELISA, Western blot, immunofluorescence (IFA) and immunohistochemistry (IHC). We have also used the anti-CP mAb, 5.5G9, in IHC studies to show that capsid antigen is persistently expressed 30 days post-infection in cells with macrophage morphology in a mouse model of chronic CHIKV disease. These antibodies may thus represent useful tools for further research including investigations on the structure and function of CHIKV CP, and as valuable reagents for CHIKV detection in a range of settings.

4.2. BODY OF TEXT

Chikungunya virus (CHIKV) is the etiological agent of CHIK fever first described in 1952 during an epidemic in Tanzania, East Africa (Lumsden, 1955; Robinson, 1955). CHIKV belongs to the *Alphavirus* genus within the *Togaviridae* family and is an enveloped, single-stranded positive-sense RNA virus (Strauss and Strauss, 1994b). The 11.5 kb alphavirus genome is capped at its 5′ end and polyadenylated at its 3′ end, and encodes for four non-structural proteins (nsP1 to nsP4) and five structural proteins (capsid, E3, E2, 6K and E1) (Strauss and Strauss, 1994b).

CHIKV is transmitted to humans by *Aedes aegypti*, and recently also *Aedes albopictus*, mosquitoes. Acute CHIKV disease is characterized by a rapid onset of fever, myalgia, and often a rash (usually maculopapular), with chronic disease characterised by episodic, often debilitating, polyarthralgia/polyarthritis (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Suhrbier *et al.*, 2012). The largest epidemic of CHIKV disease ever reported began in 2004 and has since been responsible for up to 6.5 million human cases primarily in Africa and Asia, with imported cases reported in over 40 countries (Munasinghe *et al.*, 1966; Lam *et al.*, 2001; Renault *et al.*, 2007; Rezza *et al.*, 2007; Grandadam *et al.*, 2011; Suhrbier *et al.*, 2012; Horwood *et al.*, 2013; Van Bortel *et al.*, 2014). The continued activity of the initial epidemic in conjunction with additional emerging events has led to independent outbreaks in other parts of the globe, such as in Australasia and the Caribbean (Horwood *et al.*, 2013; Viennet *et al.*, 2013; Van Bortel *et al.*, 2014). During the recent epidemic, CHIKV was also clearly associated with occasional severe disease manifestations and mortality, the latter primarily amongst elderly patients with co-morbidities and the very young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010).

The alphavirus capsid protein (CP) is a multifunctional protein that has been shown to act as a serine protease for self-cleavage, bind viral genomic RNA and other CP molecules during nucleocapsid formation, and interact with viral spike proteins during virion formation and egress (Choi *et al.*, 1991). The CP of CHIKV consists of 261 amino acids that form two major domains. The N-terminal domain has a high degree of positive charge implicated in non-specific RNA binding, while the C-terminal domain harbours the globular protease and the binding site for the spike protein (Hong *et al.*, 2006).

The re-emergence of CHIKV, attributable in large part to a mutation allowing efficient transmission by *Aedes albopictus*, and the current risk it poses to human health, has prompted the demand for new diagnostic and research reagents. Herein we report the generation and characterization of the first monoclonal antibodies (mAbs) to the CP of CHIKV and describe their use in a variety of assays.

CHIKV isolates obtained for these studies included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186); CHIKV Asian, Thailand strain ($CHIKV_{THAI}$) (GenBank ID: FJ457921) and CHIKV Asian, East Timor strain ($CHIKV_{ET}$). To generate mAbs against CHIKV viral proteins, BALB/c mice 6-8 weeks of age were immunized with purified inactivated antigen ($CHIKV_{MAU}$), challenged with live virus ($CHIKV_{THAI}$), followed by a final

boost 20 months later with inactivated antigen ($CHIKV_{THAI}$) four days prior to fusions for hybridoma production as described previously (Goh *et al.*, 2013). Hybridomas were screened for production of CHIKV-reactive antibodies using fixed-cell ELISA and positive hybridoma cultures were cloned twice by limit dilution as previously described (Hall *et al.*, 1988; Clark *et al.*, 2007). Eleven hybridomas secreting antibodies reactive to CHIKV proteins were expanded in Hybridoma SFM (Gibco, Life Technologies) with 20% FBS at 37 °C with 5% CO_2, before being weaned off all FBS for the harvesting and clarification of mAbs as culture fluid. Reactivity of these mAbs (1.7B2, 4.1H11, 4.8E2, 4.10A11, 5.1B12, 5.2F8, 5.2H7, 5.4G8, 5.5A11, 5.5D11 and 5.5G9) to various CHIKV strains and related alphaviruses were determined by fixed-cell ELISA (Table 4.1).

Each mAb recognized the three CHIKV strains used in this study ($CHIKV_{MAU}$, $CHIKV_{THAI}$ and $CHIKV_{ET}$) with similar intensity in ELISA, suggesting the epitopes are highly conserved amongst these strains. However, the varying degree of reactivity between each mAb to the CHIKV antigens, as measured by optical density reading at 405nm (OD_{405nm}), is likely due to their recognition of different binding sites on the CP and variation in the binding affinity between individual mAbs. To further assess their reactivity towards other closely-related alphaviruses, each mAb was also tested against Ross River virus (RRV) T48 strain (GenBank ID: GQ433359); Semliki Forest virus (SFV) (GenBank ID: NC_003215) and Sindbis virus (SINV) MRE16 strain (GenBank ID: AF492770). Three of the mAbs, 5.2H7, 5.5D11 and 5.5G9, reacted with antigens of SFV, RRV and SINV in ELISA, while the remaining eight mAbs recognized SFV and/or RRV but not SINV (Table 4.1). The isotype of each mAb was also determined using the Mouse Typer isotyping kit (Bio-Rad) according to the manufacturer's instructions and found to be either IgG_1 or IgG_{2A} (Table 4.1). When tested for viral neutralizing activity in a micro-neutralization assay (Goh *et al.*, 2013), none of the mAbs neutralized any of the three CHIKV strains *in vitro* (data not shown).

Table 4.1. Reactivity of CHIKV CP-specific mAbs towards various CHIKV strains and other alphaviruses in ELISA.

Monoclonal Antibody *	Reactivity in fixed-plate ELISA					
	CHIKV (MAU)	CHIKV (THAI)	CHIKV (ET)	RRV (T48)	SFV	SINV (MRE16)
5.2 H7 $_{IgG1}$	++++	++++	+++++	+++++	+++++	++++
5.5 D11 $_{IgG1}$	++++	++++	++++	+++++	+++++	++++
5.5 G9 $_{IgG2A}$	+++++	+++++	+++++	+++++	+++++	+++++
1.7 B2 $_{IgG1}$	+++++	+++++	+++++	++	-	-
4.1 H11 $_{IgG1}$	+++++	+++++	+++++	++	+	-
5.1 B12 $_{IgG2A}$	+++++	+++++	+++++	+++	+++	-
5.5 A11 $_{IgG1}$	+++++	++++	+++++	++	+++++	-
4.8 E2 $_{IgG2A}$	+++	+++	+++	++	+++	-
4.10 A11 $_{IgG1}$	+++	+++	+++	++	+++	-
5.2 F8 $_{IgG2A}$	+++	++	+++	++	++	-
5.4 G8 $_{IgG1}$	+++	++	++	++	++	-
G8 $_{IgG2A}$ †	+	+	+	+++++	+++++	-
2F2 $_{IgG1}$ ‡	-	-	-	-	-	++++

* The optimal mAb dilution producing the maximum mean OD_{405nm} reading on $CHIKV_{MAU}$ antigen was determined empirically for each mAb and used for assessment for other virus strains. Scoring: +++++, OD > 1.0; ++++, OD = 0.75 to 1.0; +++, OD = 0.5 to 0.75; ++, OD = 0.3 to 0.5; +, OD = 0.25 to 0.3.

† MAb G8 was generated to the E1 protein of RRV and is cross-reactive with CHIKV and SFV (Oliveira *et al.*, 2006).

‡ MAb 2F2, generously provided by Associate Professor Graham Burgess (TropBio Pty Ltd., Townsville, Queensland, Australia), was previously raised to the Australian prototype strain MRM39, and has been shown to be SINV-specific.

To determine their viral protein specificity, each mAb was assessed for specificity against CHIKV antigens in infected Vero cell lysate by Western blot (Goh *et al.*, 2013). $CHIKV_{MAU}$ antigens were prepared in 4 X NuPAGE LDS sample buffer (Invitrogen) and heated at 95 °C for 5 min. For reduced antigens, 10 mM dithiothreitol (DTT) was added prior to heating. The proteins were resolved on 4-12% Bis-Tris precast SDS-PAGE gels (Invitrogen), transferred onto Hybond C nitrocellulose membranes (GE Healthcare), immune-stained and developed as previously described (Clark *et al.*, 2007). To assess the glycosylation status of the target antigen, lysates were treated with PNGase F (Sigma-Aldrich) according to the manufacturer's instructions, prior to analysis by Western blot. All antibodies reacted to a protein band of ~36 kDa, in samples that were reduced or unreduced, as well as PNGase F-

treated and untreated material, consistent with recognition of the unglycosylated CHIKV CP (Fig. 4.1a).

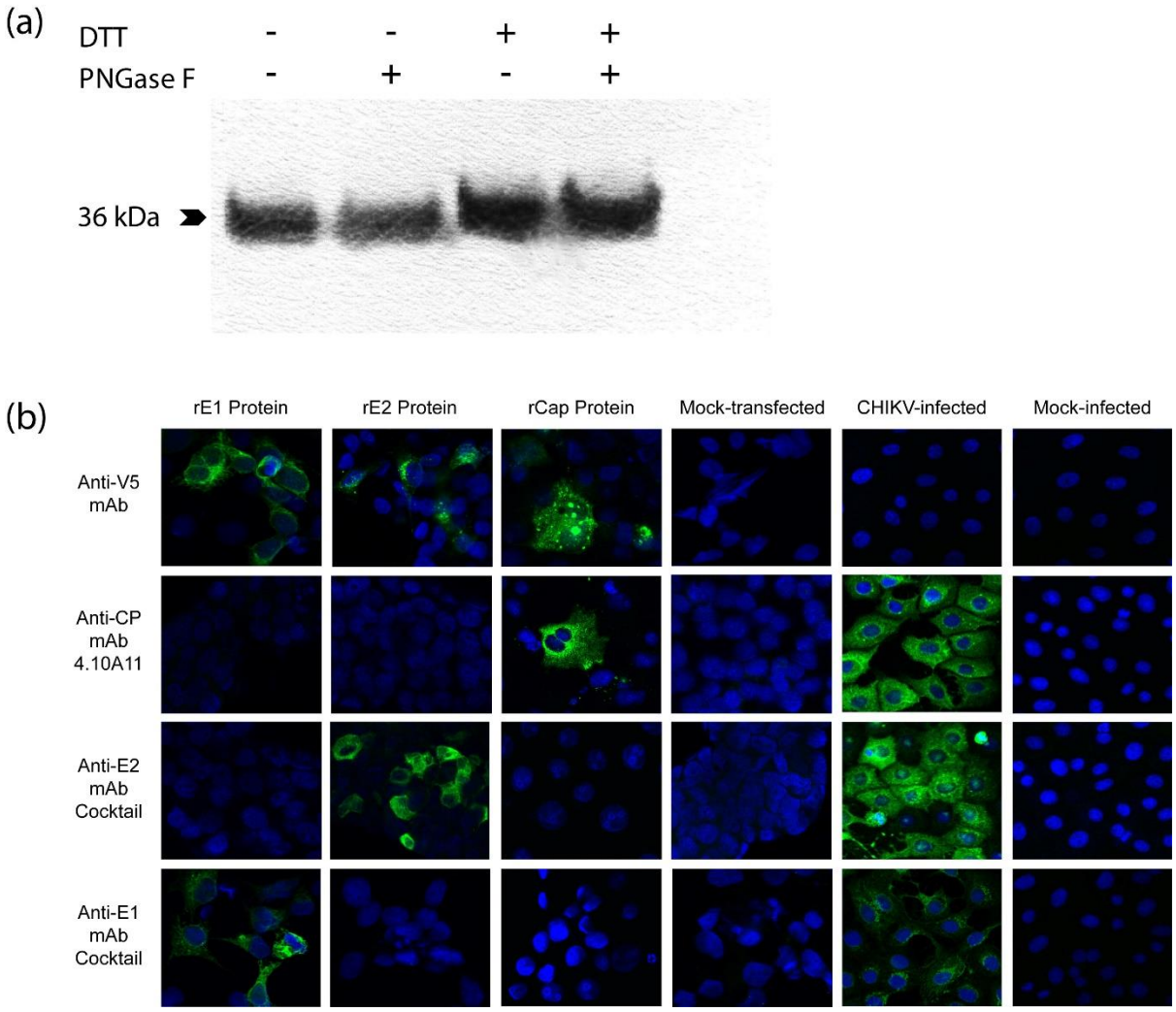

Fig. 4.1. Monoclonal antibody reactivity in Western blot and IFA. (a) Representative mAb 4.10A11 reactions against boiled, reduced (DTT+) or unreduced (DTT-) lysates of $CHIKV_{MAU}$-infected C6/36 cells treated with (+) or without (-) PNGase F. (b) IFA staining of 4.10A11 against transfected/$CHIKV_{MAU}$-infected cells. Cells were probed with mAb 4.10A11 before incubation with an anti-mouse Alexa Fluro 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining. A cocktail of five mAbs generated in a previous study was used for the detection of CHIKV E2 (Goh *et al.*, 2013). B10/G8 mAbs (anti-E1 cocktail) were generated to the E1 protein of RRV and are cross-reactive with the E1 protein of CHIKV (Oliveira *et al.*, 2006). Successful expression of recombinant proteins was demonstrated using anti-V5 mAb.

Specificity towards the CHIKV CP was confirmed by testing the mAbs for reactivity with recombinant $CHIKV_{MAU}$ CP (rCap) expressed in COS-7L cells via immunofluorescence assay **(**IFA). $CHIKV_{MAU}$ CP constructs were generated by amplifying the respective CP genes from cDNA synthesized by reverse-transcription PCR of genomic RNA of $CHIKV_{MAU}$, with primer

set “CHIKV Capsid Forward pcDNA” 5′- TATATA GCTAGC ATG GAGTTCATCCCAACCCAA -3′, “CHIKV Capsid Reverse pcDNA” 5′- TATATA GGATCC ACTCCACTCTTCGGCCCC -3′, followed by ligation into a pcDNA3.1 (+) vector (Invitrogen) modified to express V5 and histidine tags at the C-terminus of the recombinant proteins. COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. Expression of recombinant E1 (rE1) and E2 (rE2) of $CHIKV_{MAU}$ was also carried out for reference controls as previously described (Goh *et al.*, 2013). Transfected COS-7L cells were fixed onto glass coverslips with 100% ice-cold acetone and incubated with selected mAbs in hybridoma culture fluid for 1 h at 37 °C. In the case of live virus infection, Vero cells were allowed to grow overnight on glass coverslips before being infected with CHIKV at an M.O.I. of 0.1 for 1 h. Cell monolayers were then washed twice with PBS and incubated at 37 °C in complete growth medium. At 24 h post-infection, Vero cells were fixed and incubated with anti-CHIKV mAbs as described above. Coverslips were then stained, mounted and imaged as described by Goh *et al.* (2013). All 11 mAbs reacted with cells expressing rCap or cells infected with CHIKV, but not the mock-infected/transfected cells or those expressing CHIKV rE1 or rE2 (Fig. 4.1b & 4.S1).

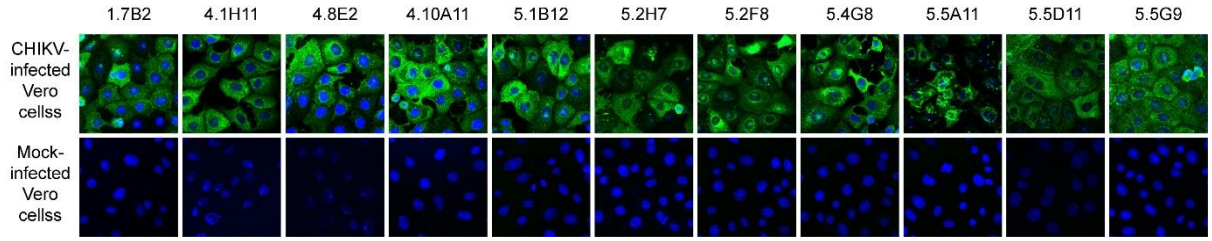

Fig. 4.S1. Monoclonal antibody reactivity by IFA with acetone-fixed monolayers of $CHIKV_{MAU}$-infected Veros cells. Cells were probed with respective mAbs before incubation with an anti-mouse Alexa Fluro 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining.

To assess the use of the CHIKV CP-specific mAbs to detect CHIKV in tissues samples, IHC was performed, as previously described in details (Goh *et al.*, 2013), on formalin-fixed, paraffin-embedded samples previously prepared from feet of $IRF3/7^{-/-}$ mice infected with $CHIKV_{REUNION}$ (Rudd *et al.*, 2012) and from wild-type (WT) mice 30 days post-infection. Briefly, deparaffinized sections were subjected to antigen retrieval by heating at 95 °C in a citrate-buffer, pH 6 (Target Retrieval Solution, DAKO) for 25 min followed by a 20 min cooling period at room temperature. Following a series of blocking steps, the sections were incubated with undiluted hybridoma culture supernatant of mAb 5.5G9 at 4 °C overnight. Preliminary studies showed that this mAb gave the most intense signal in IHC (data not shown).

Antibody binding was visualized using the anti-mouse IgG Envision kit (DAKO). Sections were counterstained with Meyer's hematoxylin, mounted and examined under a Nikon Eclipse 51E microscope. Digital micro-photographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and processed with the NIS-Elements F software. Clear staining of keratinocytes and skeletal muscle cells were observed in samples from acutely infected IRF3/7$^{-/-}$ mice with the use of mAb 5.5G9 (Fig. 4.2), consistent with previous *in situ* hybridisation studies in these interferon α/β-response deficient mice (Rudd *et al.*, 2012).

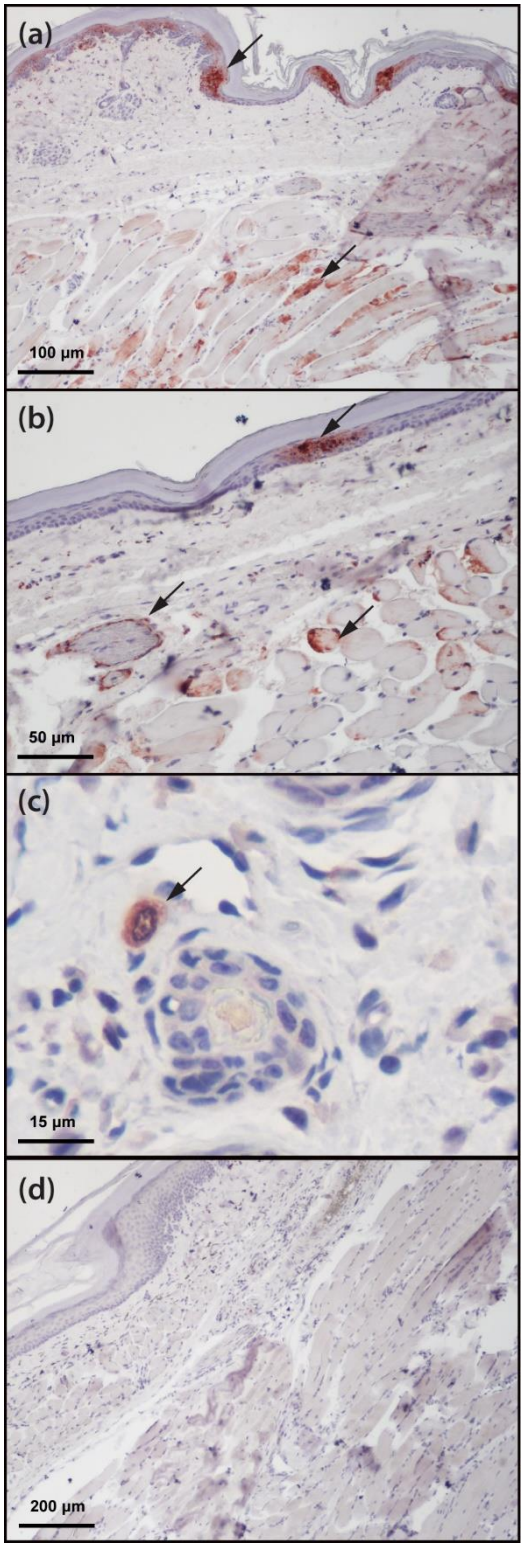

Fig. 4.2. Immunohistochemical labelling for CHIKV antigen using capsid-specific mAb 5.5G9. Labelling was observed in epidermal keratinocytes (a & b, top arrows), skeletal striated muscle cells (a & b, bottom arrows) and perineural cells (b, left arrow) in tissue sections of acutely-infected IRF3/7$^{-/-}$ mice. Macrophage-like cells within connective tissue of CHIKV-infected WT mouse feet 30 days post-infection also stained positive with 5.5G9 (c). No reactivity was observed in the uninfected control (d). Digital micrographs were capture using a

Nikon DS-Fi1 camera with a DS-U2 unit and the NIS-Elements F software and are reproduced without further manipulation.

An ongoing issue for the field of alphaviral arthritis is understanding the aetiology of chronic inflammatory disease. Persistence of CHIKV RNA and protein was reported in occasional macrophages (i) in a chronic CHIKV patient 18 months post-onset of disease in the face of a robust host immune response (Hoarau *et al.*, 2010), and (ii) in cynomolgus macaques (*Macaca fascicularis*) 44 days post-CHIKV infection by *in situ* hybridization (Labadie *et al.*, 2010), but has never been described in a mouse model, possibly due to the lack of sensitive reagents. Using the 5.5G9 mAb, we were able to detect CP antigen in scattered macrophage-like cells in connective tissue of the inoculated foot from WT mice 30 days post-infection (Fig. 4.2). This previously reported mouse model of acute infection and disease (Gardner *et al.*, 2010) thus recapitulates a key feature of chronic disease seen in humans. CP expression on day 30 - well beyond the 4-6 day viraemic period - in this model further supports the view that CHIKV antigen expression persists long term and is the likely cause of chronic inflammatory disease (Robinson, 1955; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Labadie *et al.*, 2010; Suhrbier *et al.*, 2012). In addition, the ability specifically to detect CHIKV CP positive cells in paraformaldehyde fixed, paraffin wax embedded and decalcified tissue further illustrates the utility of 5.5G9 for CHIKV research. The 5.5G9 mAb may also prove particularly useful for studies of viral persistence, as it allowed for the immune-labelling of rare cells with even low levels of CHIKV CP antigen in fixed tissue sections. CHIKV is a biosafety level 3 organism (thus tissue samples must be fixed prior to removal from the biosafety level 3 facility), with joints - necessitating decalcification - and associated tissues often the focus of research for this arthritogenic alphavirus.

In this paper, we report the first mAbs generated to the CHIKV CP, and demonstrate their reactivity in ELISA, Western blot and IFA. Our findings suggest these mAbs represent useful research tools and have strong potential in a wide variety of applications. In addition, we have shown that CHIKV antigen can be detected in infected mouse tissue samples by mAb 5.5G9 in IHC. This identifies a further application for these reagents as specific tools for the study of CHIKV pathogenesis. The mAbs generated in this study also recognized different strains of CHIKV ($CHIKV_{MAU}$, $CHIKV_{THAI}$ and $CHIKV_{ET}$) representing the two major global lineages of the virus (Asian and East/Central/South African) (Schuffenecker *et al.*, 2006). Furthermore, the three mAbs (5.2H7, 5.5D11 and 5.5G9) that also reacted strongly with the non-CHIKV

alphaviruses tested here will also be useful research tools for studying CP in related alphaviruses. The mAbs described in this paper are available from the authors upon request.

4.3. ACKNOWLEDGEMENTS

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CHAPTER 5: THE CHIKUNGUNYA VIRUS CAPSID PROTEIN CONTAINS LINEAR B CELL EPITOPES IN THE N- AND C-TERMINAL REGIONS THAT ARE DEPENDENT ON AN INTACT C-TERMINUS FOR ANTIBODY RECOGNITION.

This chapter has been submitted to Virology as **Goh, L. Y., Hobson-Peters, J., Prow, N. A., Baker, K., Piyasena, T. B., Taylor, C. T., Rana, A., Hastie, M. L., Gorman, J. J. and Hall, R. A.** (2014). The chikungunya virus capsid protein contains linear B cell epitopes in the N- and C-terminal regions that are dependent on an intact C-terminus for antibody recognition. *Virology*.

5.1. ABSTRACT

Chikungunya virus (CHIKV) is an arthropod-borne agent that causes severe arthritic disease in humans and is considered a serious health threat in areas where competent mosquito vectors are prevalent. CHIKV has recently been responsible for several millions of cases of disease, involving over 40 countries. The recent re-emergence of CHIKV and its potential threat to human health has stimulated interest in better understanding of the biology and pathogenesis of the virus, and requirement for improved treatment, prevention and control measures. In this study, we mapped the binding sites of a panel of eleven monoclonal antibodies (mAbs) previously generated towards the CP of CHIKV. Using N- and C-terminally truncated recombinant forms of the CHIKV CP, two putative binding regions, between residues 1 to 35 and 140-210, were identified. Competitive binding also revealed that five of the CP-specific mAbs recognized a series of overlapping epitopes in the latter domain. We also identified a smaller, N-terminally truncated product of native CP that may represent an alternative translation product of the CHIKV 26S RNA and have potential functional significance during CHIKV replication. Our data also provides evidence that the C-terminus of CP is required for authentic antigenic structure of CP. This study shows that these anti-CP mAbs will be valuable research tools for further investigating the structure and function of the CHIKV CP.

5.2. INTRODUCTION

Alphaviruses are spherical, enveloped, positive-sense single-stranded RNA viruses responsible for several globally significant human and animal diseases. Alphavirus members include

Sindbis virus (SINV), Semliki Forest virus (SFV), Ross River virus (RRV), the Western, Eastern and Venezuelan equine encephalitis viruses, as well as Chikungunya virus (CHIKV), all of which are transmitted by mosquitoes. CHIKV infection is characterized by onset of fever, headache, fatigue, nausea, myalgia and maculopapular rash, often followed by severe acute and/or chronic polyarthralgia (Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Suhrbier *et al.*, 2012). Since its re-emergence in the early 2000s, CHIKV has been responsible for a succession of unprecedented outbreaks causing up to 6.5 million human infections in over 40 countries in East Africa, the Indian Ocean islands, several regions of South Asia, and most recently in Europe, Oceania and the Caribbean regions (Munasinghe *et al.*, 1966; Lam *et al.*, 2001; Renault *et al.*, 2007; Rezza *et al.*, 2007; Grandadam *et al.*, 2011; Suhrbier *et al.*, 2012; Horwood *et al.*, 2013; Van Bortel *et al.*, 2014). It has also been recently associated with severe disease manifestations, and mortality in some cases, largely amongst elderly patients with co-morbidities and the very young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010).

Similar to other alphaviruses, CHIKV has a ~11.5 kb RNA genome that is capped at its 5′ end and polyadenylated at its 3′ end. The genome encodes four non-structural proteins, nsP1 to nsP4, and five structural proteins: capsid, E3, E2, 6K and E1 (Strauss and Strauss, 1994b). Studies have shown that the alphavirus capsid protein (CP) is multifunctional and plays a crucial role in the assembly and budding of alphaviruses. It is capable of self-cleavage prior to the recognition and binding of genomic RNA (Choi *et al.*, 1991). Apart from its primary role of forming the nucleocapsid, the CP has been shown to have inhibitive and/or regulative functions in regard to host cell transcription, viral replication, as well as host and viral protein synthesis (Elgizoli *et al.*, 1989; Aguilar *et al.*, 2007). The CP of alphaviruses, as described in a SINV model, is organized into three separate regions – I, II and III – each with their respective functions (Hong *et al.*, 2006). As shown in Figure 5.1, the unconserved N-terminal domain of the alphavirus CP has a high degree of positive charge implicated in non-specific RNA binding, while the highly-conserved C-terminal region harbours a globular chemotrypsin-like serine protease and contains the binding site for the spike protein. A recent study by Thomas *et al.* (2013) has also identified functional nuclear localization and export signals (NLS/NES) within the CHIKV CP. Furthermore, previous reports on the discovery of nucleolar targeting signals in SFV CP suggest that these signals are responsible for the karyophilic properties of the protein (Nigg *et al.*, 1991; Favre *et al.*, 1994; Jakob, 1994). NLS sequences in forms of synthetic peptides have been used to demonstrate efficient transport of the CP into the nucleus of both

higher and lower eukaryotic target cells (Favre *et al.*, 1994). Nonetheless, a functional role has yet to be attributed to this putative intracellular transport of the CP during infection.

The CP is a critical component of the assembly of alphaviruses. To date, alphaviral CP research is mainly based on models of SINV and SFV, exact roles and/or functions of the CHIKV CP has largely been assumed to be similar to that of its closely-related viruses. However, the re-emergence of CHIKV and its potential threat to human health has demanded for a more detailed understanding of the virus. Herein we report the use of a series of truncated recombinant proteins derived from the CHIKV CP, mass spec analysis of native CHIKV CP and competitive binding studies to map the binding sites of a panel of eleven monoclonal antibodies (mAbs) to antigenic domains at the N-terminal region and the C-terminal half of the protein.

5.3. MATERIAL AND METHODS

5.3.1. Cell and virus culture

C6/36 (*Aedes albopictus* mosquito) cells were propagated in RPMI 1640 supplemented with 2% fetal bovine serum. Cultures were passaged by dissociating the cell monolayer from the flask with trypsin/PBS and were incubated at 28 °C. Vero and COS-7L (African green monkey kidney) cell lines were cultured in DMEM and RPMI 1640, respectively, supplemented with 5% fetal bovine serum (FBS) during proliferation and 2% FBS for maintenance. Mammalian cells were passaged by dissociating the surface monolayer from the flask with trypsin/EDTA and were cultured at 37 °C with 5% CO_2. Hybridoma cells were expanded in Hybridoma SFM (Gibco, Life Technologies) with 20% FBS at 37 °C with 5% CO_2, before being weaned off all FBS for the harvesting of mAbs in culture fluid. All cell cultures were supplemented with 50 U penicillin mL^{-1}, 50 µg streptomycin mL^{-1} and 2 mM L-Glutamine (Gibco, Life Technologies).

Viruses used for the infection of C6/36 cells included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186) and RRV T48 strain (RRV_{T48}) (GenBank ID: GQ433359). For harvesting of virus stocks, cells were infected at an M.O.I. of 0.1 for 1 h, washed thrice with PBS and incubated for a further 48-72 h before culture supernatants were clarified by centrifugation at 12,000 x g for 10 min at 4 °C and stored at -80 °C. To obtain crude cell lysates, mock and/or virus-infected C6/36 cell monolayers were incubated in a similar manner before cells were rinsed in PBS and disrupted by sonication in the presence of BS9 lysis buffer (120 mM NaCl, 50 mM H_3BO_3, 1% Triton X-100 and 0.1% SDS, pH 9.0). Lysates were clarified as mentioned above and stored at -20 °C (Clark *et al.*, 2007).

5.3.2. Cloning and expression of CHIKV CP truncations

CHIKV CP truncation constructs (Figure 5.1, bottom) were generated by amplifying the respective CP genes from cDNA synthesized by reverse-transcription PCR of genomic RNA of $CHIKV_{MAU}$, either by pairing the CHIKV Capsid Forward PCDNA primer with one of the CHIKV Capsid N1-6 Reverse PCDNA primers, or the CHIKV Capsid Reverse PCDNA primer with one of the CHIKV Capsid C1-6 Forward PCDNA primers (Table 5.1). Following that, inserts were ligated into a pcDNA3.1 (+) vector (Invitrogen) modified to express V5 and histidine tags at the C-terminus of the recombinant proteins. COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were harvested 48 h post-transfection by addition of BS9 lysis buffer and clarified by centrifugation (Hobson-Peters *et al.*, 2008).

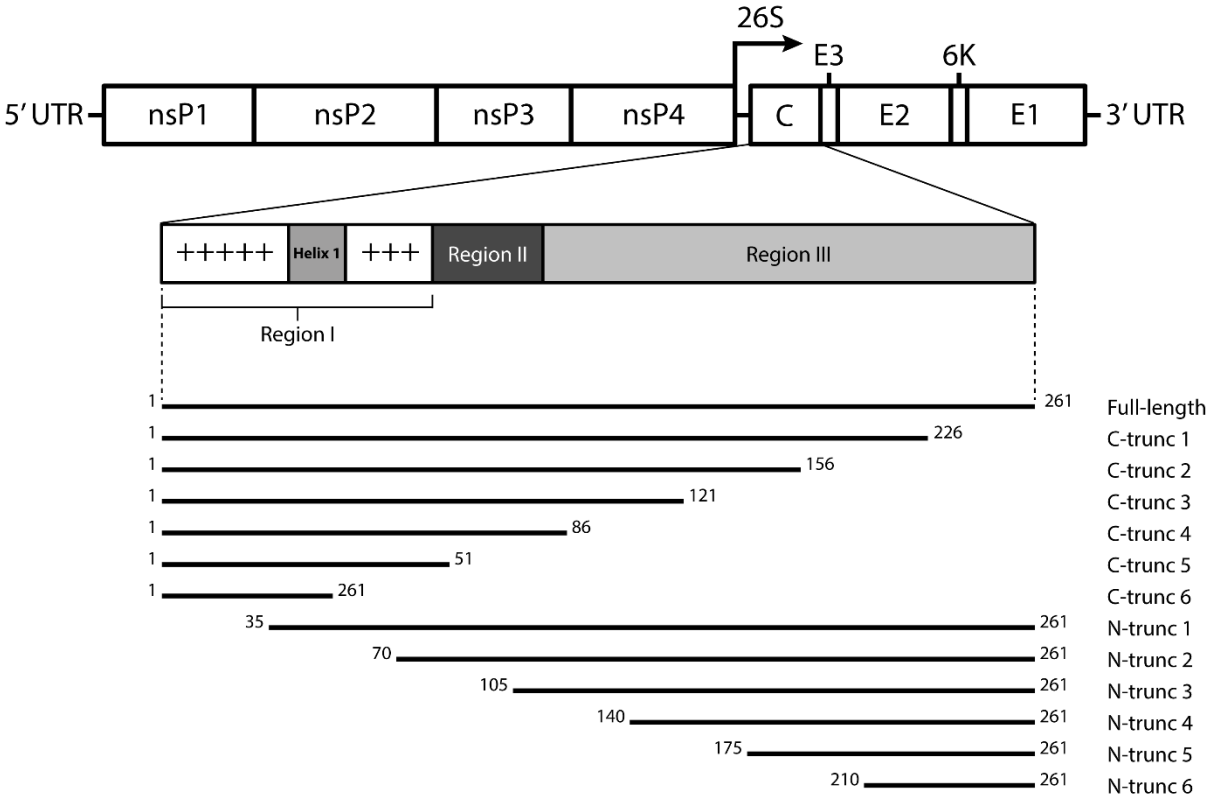


Figure 5.1. Schematic representation of the alphavirus CP within its RNA genome. The alphavirus CP is segmented into three separate regions, based on the prototype SINV CP model - adapted from Hong *et al.* (2006). Region I has a high degree of positive charge associated with non-specific binding of the genomic viral RNA. Located within the same region, Helix 1, a sequence of uncharged amino acids, is suggested to be involved in the interactions during nucleocapsid core formation and its stabilization. Region II contains the minimum sequence required for specific RNA binding, while the C-terminal protease domain (region III) forms capsomeres in the nucleocapsid core and interacts with the E2 glycoprotein. The bottom part of the figure depicts the locations of the C- or N-terminal truncations within the full-length CP. Truncations were made in 35-amino acid increments.

Table 5.1. Nucleotide sequences of CHIKV CP truncation primers.

Primer code	Sequence (5′ to 3′)	Tm (°C)	Expected size (bp)
CHIKV Capsid F PCDNA	TATATAGCTAGCATGGAGTTCATCCCAACCCAA	76.7	-
CHIKV Capsid N1 R PCDNA	TATATAGGATCCGGCCACCACGCGTCCCT	72.3	678
CHIKV Capsid N2 R PCDNA	TATATAGGATCCGTGGTGCCAGTTGTAGTAC	55.3	573
CHIKV Capsid N3 R PCDNA	TATATAGGATCCCCGCTTAAAGGCCAGTTT	61.7	468
CHIKV Capsid N4 R PCDNA	TATATAGGATCCACCTTCGTGCTTGACTTC	57.8	363
CHIKV Capsid N5 R PCDNA	TATATAGGATCCCTGCTTCTTTTGATTTGTG	55.9	258
CHIKV Capsid N6 R PCDNA	TATATAGGATCCCAGTTTATTAACTGCTGAGATC	55.3	153
CHIKV Capsid R PCDNA	TATATAGGATCCACTCCACTCTTCGGCCCC	74.8	-
CHIKV Capsid C1 F PCDNA	TATATAGCTAGCATGAGGCAAGCTGGGCAAC	67.6	678
CHIKV Capsid C2 F PCDNA	TATATAGCTAGCATGAAGCAAAAACAACAGGC	74.8	573
CHIKV Capsid C3 F PCDNA	TATATAGCTAGCATGTGCATGAAAATCGAAAAT	60.7	468
CHIKV Capsid C4 F PCDNA	TATATAGCTAGCATGAAGGGGACCATCGATAA	67.7	363
CHIKV Capsid C5 F PCDNA	TATATAGCTAGCATGTCGAAGTTCACCCATGA	65.6	258
CHIKV Capsid C6 F PCDNA	TATATAGCTAGCATGGGGGACAGCGGCAGA	71.6	153

5.3.3. Immunofluorescence assay (IFA)

Transfected COS-7L cells were fixed onto glass coverslips with 100% ice-cold acetone and incubated with selected mAbs in hybridoma culture fluid at a 1/20 dilution for 1 h at 37 °C. Coverslips were then washed and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:500 in blocking buffer (5% BSA in PBS) for 1 h at 37 °C, followed by Hoechst 33342 stain (1:1,000 in PBS, Invitrogen) for 5 min. Coverslips were mounted with ProLong Gold Anti-Fade reagent (Invitrogen) and imaged using a Zeiss LSM 510 META confocal microscope.

5.3.4. Western/dot blot

CHIKV antigens were prepared as transfected COS-7L cell lysates as described by Setoh *et al.* (2011). Reduction and carboxymethylation of antigens were carried out as previously described (see section 3.3.7.). Briefly, lysates were diluted in Tris-HCl and reduced with 10 mM dithiothreitol. Samples were then degassed with streaming nitrogen and heated to 95 °C. The reduced lysates were cooled and iodoacetic acid was added before being subjected to a second round of degassing, followed by incubation at 37 °C in the dark. Antigens that were to be resolved on 4-12% Bis-Tris precast SDS-PAGE gels (Invitrogen), were prepared in 4 X NuPAGE LDS sample buffer (Invitrogen) and heated to 95 °C for 5 min prior to electrophoresis. The separated proteins were then transferred onto Hybond C nitrocellulose

membranes (Amersham) and immune-stained as previously described (Clark *et al.*, 2007). For dot-blotting, treated or untreated antigen samples were carefully spotted directly onto Hybond C nitrocellulose membranes and allowed to dry for 5-10 min. Membranes were then blocked with TENTC blocking buffer (0.05 M Tris-HCl pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.05% (v/v) Tween 20, 0.2% (w/v) casein) for 1 h at room temperature prior to the addition of primary antibodies diluted 1/20, unless otherwise stated, in blocking buffer. After incubation for another hour, membranes were washed thrice with 0.1% Tween-20 in PBS (PBS/T wash buffer) and bound antibodies were detected with a HRP-conjugated goat anti-mouse IgG diluted 1:4,000 in blocking buffer. The blots were incubated for a further 1 h before being washed three times with PBS/T wash buffer. Finally, blots were developed in DAB substrate solution (1.5 mM 3,3′-diaminobenzidine, 0.06% (v/v) H_2O_2 in PBS, pH 7.2) for 15 min before reactions were terminated by rinsing with PBS or ddH_2O.

5.3.5. Competitive binding ELISA

Competitive binding between anti-CHIKV CP mAbs were assessed as described previously (Hall *et al.*, 2009). Briefly, purified mAbs were biotinylated using the BiotinTag kit (Sigma), according to manufacturer's instructions. Competitive binding ELISAs were performed in 96 well plates coated with lysates of $CHIKV_{MAU}$-infected C6/36 cells diluted 1/500 in coating buffer (0.05M sodium carbonate/bicarbonate, pH 9.6). After washing, a pre-defined optimal saturating concentration of each of the unlabelled mAbs was added for 1 h at 28 °C. Without washing, a pre-defined optimal non-saturating dilution of each biotin-labelled 'competitor' mAb (40 ng/mL) was added for 1 h at 28 °C. After washing six times with PBS/T, horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen) was added and incubated for 30 min. The wells were washed prior to incubation with ABTS substrate solution. Uninfected C6/36 cell lysates were used as coating antigen to determine background cut-off values, while 4G2, a pan-reactive mAb targeting the flavivirus envelope protein, was used as the negative antibody control.

5.3.6. Microsphere immunoassay

A series of 20-mer peptides, with 10-residue overlaps, encompassing the entire CHIKV CP were designed (see Table 5.2) and commercially synthesized (Mocell Biotech). Each peptide was coupled to carboxylated polystyrene beads using the Bio-Plex amine coupling kit (Bio-Rad) according to the manufacturer's instructions. The coupled beads were then multiplexed

by diluting in microsphere immunoassay (MIA) buffer (1% BSA, 0.05% ProClin 300 (Supelco) in PBS) and distributed to give approximately 2,500 of each beadset (~100 µL) per well of a MultiScreen filter plate (Merck Millipore). Each anti-CHIKV CP mAb was then tested against the multiplexed beadsets by incubation for 45 min with shaking at room temperature. The wells were then washed three times with PBS/T wash buffer, prior to the addition of R-Phycoerythrin donkey anti-mouse IgG (Jackson ImmunoResearch). Following that, the wells were incubated and washed as previously, after which the beads were resuspended in MIA buffer. The Bio-Plex 200 (Luminex, Bio-Rad) was then utilized to read the plate, measuring the mean fluorescence intensity for each beadset in their respective wells.

5.3.7. Immunoprecipitation of CHIKV CP

Protein pull-downs were performed on clarified lysates of $CHIKV_{MAU}$-infected C6/36 cells using Dynabeads Protein G (Novex, Life Technologies) according to manufacturer's instructions. Boiled and/or reduced samples were then resolved via SDS-PAGE as described above (see section 5.3.4.) prior to incubation of the gel in Coomassie blue stain (1% (w/v) Coomassie R250, 10% glacial acetic acid, 40% methanol in ddH_2O) at room temperature for 30 min before being rinsed twice with destaining solution (10% glacial acetic acid, 40% methanol in ddH_2O). Gel was then incubated in destaining solution overnight with rocking.

5.3.8. Mass spectrometry analysis

Individually stained bands were excised reduced, alkylated and subjected to in-gel tryptic digestion as described in detail previously (Hastie *et al.*, 2012). Acidified digests were subjected to NanoHPLC-MS/MS analysis using a nanoAcquity nanoHPLC system (Waters) interfaced with a linear ion-trap (LTQ)-Orbitrap Elite hybrid mass spectrometer (Thermo Fischer Scientific). Digests were loaded onto a 5 µ Symmetry 180 µm × 20 mm C18 trap column (Waters) at 15 µL/min in 98% solvent A (0.1% (v/v) aqueous formic acid) and 2% solvent B (0.1% (v/v) formic acid in 100% acetonitrile) for 3 min at 22 °C then switched in-line with a pre-equilibrated analytical column (BEH130 C18 1.7 µm, 75 µm × 200 mm, Waters) at a flow rate of 0.3 µL/min and 98% solvent A, 2% solvent B. Peptides were separated at 35 °C using a sequence of linear gradients: starting from 5% B over 1 min, to 40% B over 29 min, and finally, to 95% B over 4 min, before holding the column at 95% B for a further 4 min. Eluates from the analytical column were then introduced into the LTQ-Orbitrap Elite throughout the entire run via a Nanospray Flex Ion Source (Thermo Fisher Scientific)

containing a 10 µm P200P coated silica emitter (New Objective). Typical spray voltage was 1.8 kV with no sheath, sweep or auxiliary gases; heated capillary temperature was set to 275 °C. The LTQ- Orbitrap Elite was controlled using Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific) and operated in a data-dependent acquisition mode to automatically switch between Orbitrap-MS and ion trap-MS/MS. The survey full scan mass spectra (from m/z 380-1700) were acquired in the Orbitrap with a resolving power of 120,000 after accumulating ions to an automatic gain control (AGC) target value of 1.0×10^6 charges in the LTQ. MS/MS spectra were concurrently acquired in the LTQ on the 20 most intense ions from the survey scan, using an AGC target value of 1.0×10^4. Charge state filtering, where unassigned precursors and singly charged ions were not selected for fragmentation, and dynamic exclusion (repeat count 1, repeat duration 30 sec, exclusion list size 500, exclusion duration 30 sec) were used. Fragmentation conditions in the LTQ were: 35% normalized collision energy, activation q of 0.25, 10 ms activation time, and minimum ion selection intensity of 500 counts. Maximum ion injection times were 250 ms and 100 ms for survey full scans and MS/MS scans, respectively.

5.3.9. Data analysis

Tandem mass spectra were processed using Proteome Discoverer (version 1.4, Thermo Fisher Scientific) and submitted to Mascot (version 2.5.1, Matrix Science). Fixed modification: carbamidomethyl-cysteine; variable modifications: deamidation (asparagine, glutamine) and oxidation (methionine). Enzyme: trypsin, 2 missed cleavages; MS tolerance 20 ppm; MSMS tolerance 0.6 Da using a database downloaded from UniprotKB on the 21 July 2014 consisting of the reference proteome for Aedes aegypti (UP000008820), Chikungunya virus (strain 37997) (UP000008450), reference proteome Chikungunya virus (strain S27-African prototype) with the sequence of the capsid protein added as a separate entry. Scaffold (4.4.1.1, Proteome Software) was used to validate Mascot protein identifications (Searle, 2010). Scaffold probabilistically validates these peptide identifications using PeptideProphet (Keller *et al.*, 2002). A cut-off of 99% +2, +3 and 5 ppm error was used to validate the peptides.

5.4. RESULTS

5.4.1. Anti-CHIKV CP mAbs recognize linear epitopes

To determine whether a panel of eleven anti-CHIKV CP mAbs bound conformational or linear epitopes, antigens in the form of crude lysates derived from CHIKV-infected C6/36 cells were

subjected to reduction with DTT and carboxymethylation of their free sulfhydryl groups so as to prevent the reformation of disulphide bonds. These antigens were separated by SDS-PAGE alongside boiled, unreduced, or boiled, reduced, uncarboxymethylated lysates prior to immunoblotting. Probing with each of the CP-specific mAbs, including representative antibody 1.7B2, revealed that all eleven antibodies recognized CP under both unreduced and reduced conditions, even after carboxymethylation (Figure 5.2), indicating that the anti-CHIKV CP mAbs recognized linear epitopes that are not dependent on disulphide bonds to provide secondary structure.

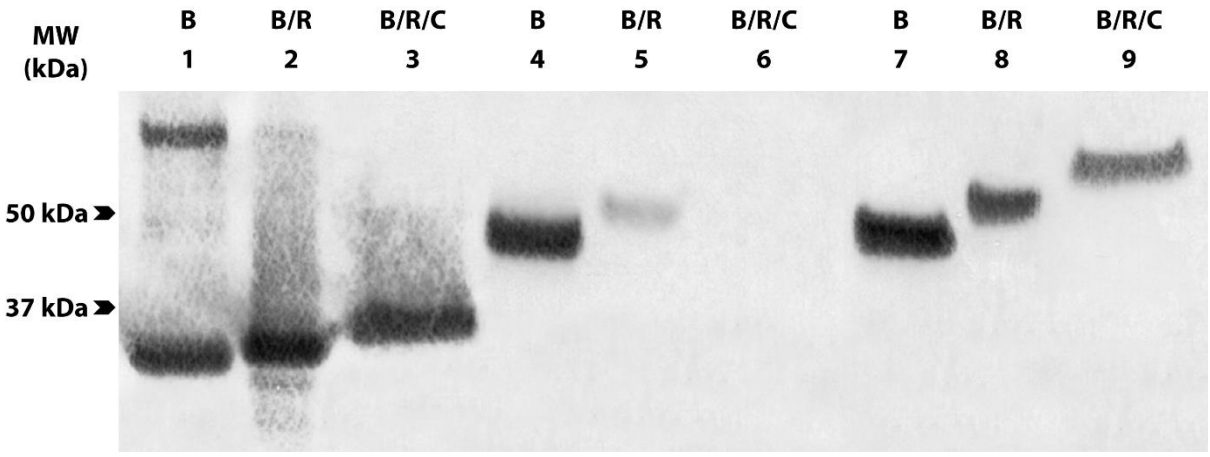


Figure 5.2. Reactivity of anti-CHIKV CP mAbs against boiled, reduced and/or carboxymethylated CHIKV cell lysates. Boiled (B), boiled and reduced (B/R) or boiled, reduced and carboxymethylated (B/R/C) lysates of CHIKV (lanes 1-3), RRV (lanes 4-6) and West Nile virus (lanes 7-9) were probed against antibodies 1.7B2 (anti-CP), RRG8 (anti-E1) and 17D7 (anti-E), respectively, in Western blot. Note that the increase in molecular weight with each treatment is an indication of successful chemical modification.

Since the Western blot results indicated that all anti-CP mAbs recognized linear epitopes, we designed a series of 20-mer synthetic peptides, with 10-residue overlaps, spanning the entire CHIKV CP to fine-map the epitopes of the anti-CHIKV mAbs. However, none of the mAbs showed any reactivity towards the synthetic peptides in ELISA, dot blot or in MIA, despite the successful detection of all control peptides epitopes by their corresponding mAbs using these assays (Table 5.2). This surprising result suggested that the binding of the CP-specific mAbs may require additional structure provided by post-translational modifications not found in synthetic peptides.

Table 5.2. Reactivity of synthetic peptides in MIA, ELISA and dot blot.

No.	Sequence	Length	Protein*	Position	Luminex	ELISA	Dot Blot
1.	MEFIPTQTFYNRRYQPRPWT	20	CHIKV CP	1-20	-	-	-
2.	NRRYQPRPWTPRPTIQVIRP	20	CHIKV CP	11-30	-	-	-
3.	PRPTIQVIRPRPRPQRQAGQ	20	CHIKV CP	21-40	-	-	-
4.	RPRPQRQAGQLAQLISAVNK	20	CHIKV CP	31-50	-	-	-
5.	LAQLISAVNKLTMRAVPQQK	20	CHIKV CP	41-60	-	-	-
6.	LTMRAVPQQKPRRNRKNKKQ	20	CHIKV CP	51-70	-	-	-
7.	PRRNRKNKKQKQKQQAPQNN	20	CHIKV CP	61-80	-	-	-
8.	KQKQQAPQNNTNQKKQPPKK	20	CHIKV CP	71-90	-	-	-
9.	TNQKKQPPKKKPAQKKKKPG	20	CHIKV CP	81-100	-	-	-
10.	KPAQKKKKPGRRERMCMKIE	20	CHIKV CP	91-110	-	-	-
11.	RRERMCMKIENDCIFEVKHE	20	CHIKV CP	101-120	-	-	-
12.	NDCIFEVKHEGKVTGYACLV	20	CHIKV CP	111-130	-	-	-
13.	GKVTGYACLVGDKVMKPAHV	20	CHIKV CP	121-140	-	-	-
14.	GDKVMKPAHVKGTIDNADLA	20	CHIKV CP	131-150	-	-	-
15.	KGTIDNADLAKLAFKRSSKY	20	CHIKV CP	141-160	-	-	-
16.	KLAFKRSSKYDLECAQIPVH	20	CHIKV CP	151-170	-	-	-
17.	DLECAQIPVHMKSDASKFTH	20	CHIKV CP	161-180	-	-	-
18.	MKSDASKFTHEKPEGYYNWH	20	CHIKV CP	171-190	-	-	-
19.	EKPEGYYNWHHGAVQYSGGR	20	CHIKV CP	181-200	-	-	-
20.	HGAVQYSGGRFTIPTGAGKP	20	CHIKV CP	191-210	-	-	-
21.	FTIPTGAGKPGDSGRPIFDN	20	CHIKV CP	201-220	-	-	-
22.	GDSGRPIFDNKGRVVAIVLG	20	CHIKV CP	211-230	-	-	-
23.	KGRVVAIVLGGANEGARTAL	20	CHIKV CP	221-240	-	-	-
24.	GANEGARTALSVVTWNKDIV	20	CHIKV CP	231-250	-	-	-
25.	SVVTWNKDIVTKITPEGAEEW	21	CHIKV CP	241-261	-	-	-
26.	SAAKHARKERNITGGHPVSR ‡	20	WNV_{KUN} NS5	38-57	+	+	+
27.	CTTVESHGNYSTQVGATQAG ‡	20	WNV_{NY99} E	146-165	+	+	+
28.	Full-length CHIKV CP #	261	CHIKV CP	N/A	ND	+	+

* Each synthetic peptides was screened against all eleven anti-CHIKV CP mAbs, polyclonal anti-CHIKV antibodies, as well as 5H1 (anti-$WNV_{KUN}\alpha 3$) and 17D7 (anti-WNY_{NY99} E).

‡ A "+" result represents reactivity with the peptide's respective control mAb.

ND = not determined. Full-length CHIKV CP was reactive towards all eleven anti-CHIKV CP mAbs and polyclonal anti-CHIKV antibodies in ELISA and dot blot.

We have previously shown that these mAbs react with CP treated with PNGase F (see Chapter 4), indicating that N-linked glycosylation is not required, consistent with the absence of an N-linked glycosylation site and the cytoplasmic location of the protein (Goh *et al.*, 2014). Further analysis of the CHIKV CP sequence using a prediction software, NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), revealed the presence of several potential phosphorylation sites that may participate in the structure of epitope(s) recognized by these mAbs (Figure 5.3). However, attempts to demonstrate this by dephosphorylation of CHIKV CP prior to analysis by Western blot were inconclusive.

```
RRVT48          MNYIPTQTFYGRRWRPRP-AFRPWQVPMQPTPTMVTPMLQAPDLQAQQMQQLISAVSALT 59
SFV             MNYIPTQTFYGRRWRPRP-AARPW--PLQATP--VAPVV--PDFQAQQMQQLISAVNALT 53
CHIKVMAU        MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPR--------PQRQAGQLAQLISAVNKLT 52
BFV             MDFIPTQTFYGRRWRPAP-VQRYIPQPQPPAPP-------RRRRGPSQLQQLVAALGALA 52
SINVMRE16       MNRG-FFNMLGRRPFPAPTSMWRPRRRRQAAPG------PARNGLATQIQQLTSAVNALV 53
                *:     .: .**  * *           . *            . *: ** :*:. *.

RRVT48          TKQNVKAPKGQRK-KKQQKPKEKKENQKKKPTQKKKQQQKPKPQA--KKKKPGRRERMCM 116
SFV             MRQNAIAPARPPKPKKKKTTKPKPKTQPKKINGKTQQQKKKDKQADKKKKKPGKRERMCM 113
CHIKVMAU        MR--AVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQPPKKKPAQK---KKKPGRRERMCM 107
BFV             LQPKQKQKRAQKKPKKTPPPKPKKTQKPKKPTQKK-------------KSKPGKRMRNCM 99
SINVMRE16       IGQVSRQQQPRQRPAPKPRRQPPKQQQPKPKKTKNPEKPKKKQPT---KPKPGKRQRMAL 110
                            :       :       :  . *.             * ***:* * .:

RRVT48          KIENDCIFEVKLDG-KVTGYACLVGDKVMKPAHVKGTIDNPDLAKLTYKKSSKYDLECAQ 175
SFV             KIENDCIFEVKHEG-KVTGYACLVGDKVMKPAHVKGVIDNADLAKLAFKKSSKYDLECAQ 172
CHIKVMAU        KIENDCIFEVKHEG-KVTGYACLVGDKVMKPAHVKGTIDNADLAKLAFKRSSKYDLECAQ 166
BFV             KIENDCIFPVMLDG-KVNGYACLVGDKVMKPAHVKGTIDNPELAKLTFKKSSKYDLECAQ 158
SINVMRE16       KLEADRLFDVKNEAGEVIGHALAMEGKVMKPLHVKGTIDHPVLSKLKFTKSSAYDMEFAQ 170
                *:* * :* *  :. :* *:*  : .***** ****.**:. *:** :.:** **:* **

RRVT48          IPVHMKSDASKYTHEKPEGHYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGRVVA 235
SFV             IPVHMRSDASKYTHEKPEGHYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGRVVA 232
CHIKVMAU        IPVHMKSDASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGRVVA 226
BFV             VPVCMKSDASKFTHEKPEGHYNWHHGAVQFSNGRFTIPTGSGKPGDSGRPIFDNTGKVVA 218
SINVMRE16       LPTNMRSEAFSYTSEHPEGFYNWHHGAVQYSGGRFTVPKGAGGKGDSGRPIMDNTGKVVA 230
                :*. *:*:* .:* *:***.*********:*.****:*.*:*  *******:**.*:***

RRVT48          IVLGGANEGARTALSVVTWT-KDMVTRVTPEGTEEW 270
SFV             IVLGGANEGSRTALSVVTWN-KDMVTRVTPEGSEEW 267
CHIKVMAU        IVLGGANEGARTALSVVTWN-KDIVTKITPEGAEEW 261
BFV             IVLGGANEGARTALSVVTWN-KDMVTRITPEESVEW 253
SINVMRE16       IVLGGADEGARTALSVVTWNSKGKTIKTTPEGTEEW 266
                ******:**:*********. *. . : *** : **
```

Figure 5.3. Capsid protein amino acid sequence alignment of closely-related alphaviruses. Asterisks (*), colons (:) and periods (.) indicate identical, conserved or semi-conserved substitutions, respectively. Residues highlighted in red represent potential phosphorylation sites predicted by NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), while yellow residues mark the conserved catalytic triad residues involved in the auto-proteolytic function of the CP. The teal-coloured tryptophan (position 261) is where the auto-cleavage occurs. Contributing amino acids of the predicted NLS and NES sequences are highlighted in green and blue, respectively. Red, bolded residues of the NES denote hydrophobic positions, indicating its similarity towards a non-classical NES sequence (Thomas *et al.*, 2013). The methionine (position 105) highlighted in magenta

indicates the potential alternative translation initiation site of the N-terminally truncated form of CP. Putative mAb binding domains are in bold (group 1 mAbs: 157-175; group 2 mAbs: 1-35).

5.4.2. Expression of N- and C-terminally truncated CHIKV CP

In another approach to map the epitope(s) recognized by the anti-CP mAbs, recombinant full-length and a series of N- and C-terminally truncated CP sequences were expressed in COS-7L cells. Successful expression of full length and truncated proteins C-terminally fused to a V5-His tag, was confirmed by detection with anti-V5 mAb in IFA (Figure 5.4). Most of the truncations were also recognized by anti-V5 mAb in Western blot (Figure 5.5), although the smaller protein bands were not successfully transferred to the nitrocellulose membrane due to limitation of the membrane pore size.

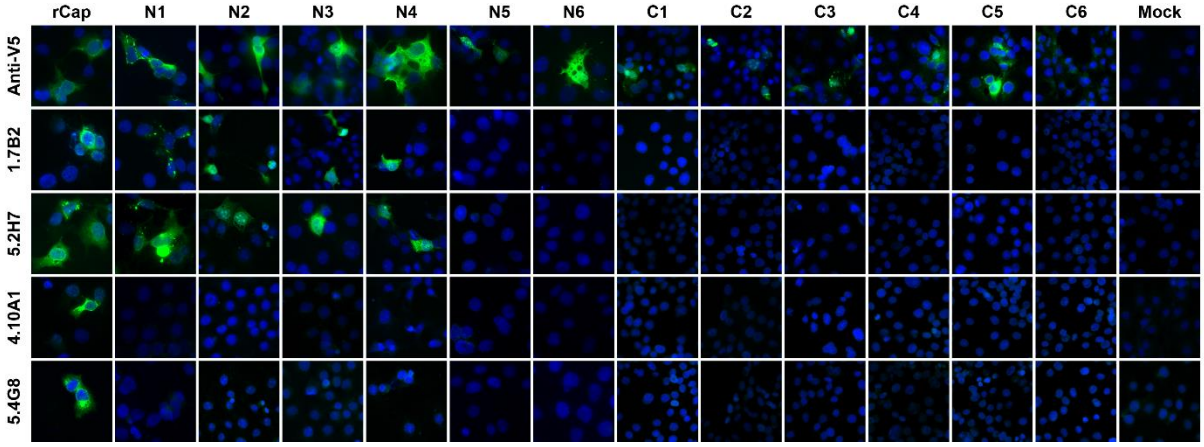


Figure 5.4. Monoclonal antibody reactivity by IFA with acetone-fixed monolayers of COS-7L cells transfected with full-length rCap or truncation constructs. Cells were probed with respective mAbs before incubation with an anti-mouse Alexa Fluro 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining. Two mAbs representing each group were chosen for this experiment: Group 1 - 1.7B2, 5.2H7, and Group 2 - 4.10A11, 5.4G8.

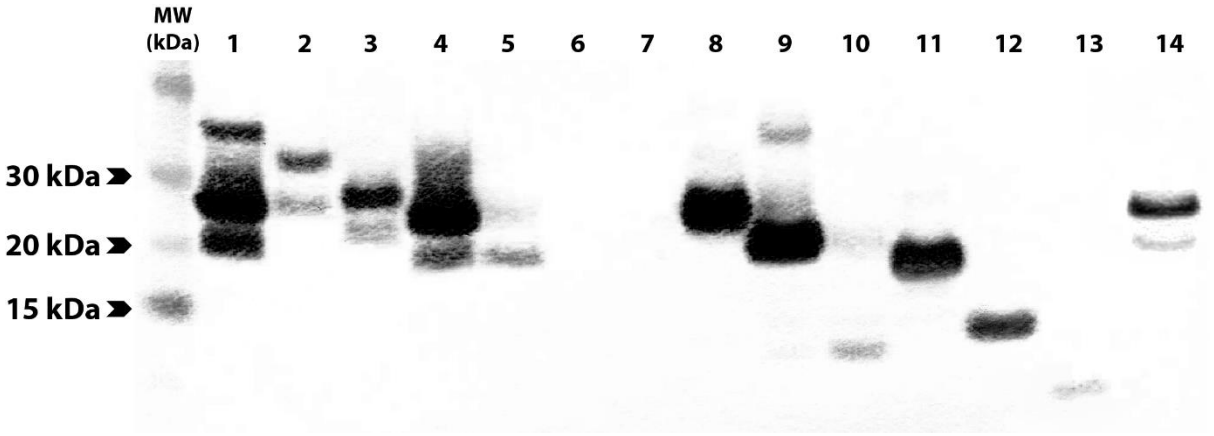


Figure 5.5. Western blot of recombinant full-length and truncated CP in lysates of transfected cells. Clarified lysates of transfected COS-7L cells were boiled and reduced with DTT prior to immunoblotting with anti-V5 mAb. Lanes 1-6: C1-6; lanes 7: mock-transfected COS-7L lysate; lanes 8-13: N1-6; and lane 14: full-length rCap.

To identify the binding regions of these mAbs in CP, each antibody was subjected to Western blot analysis against each of the twelve truncated versions of CHIKV CP. Monoclonal antibodies 1.7B2, 4.1H11, 5.2H7, 5.5D11 and 5.5G9 (designated group 1), reacted with a series of the N-truncations (N1-4), as well as the full-length protein (rCap) (Table 5.3). This demonstrated that group 1 mAbs still recognized CP in the absence of the first 140 residues (see Figure 5.1) suggesting the epitope(s) they recognize resides in the C-terminal half of the protein. Failure of the group 1 mAbs to bind CP with larger truncations of between 175 (N5) and 210 residues (N6), conservatively placed their binding site between residues 140 and 210 in CP. Meanwhile, group 2 mAbs (4.8E2, 4.10A11, 5.1B2, 5.2F8, 5.4G8 and 5.5A11) were able to detect rCap but none of the N-terminally truncated proteins (Table 5.3). This suggested that all mAbs from group 2 bound the N-terminal region of the CP, since only the first 35 residues were missing from the smallest N-truncation (N1). Similar results were observed when group 1 and 2 mAbs were tested against the N-terminally truncated recombinant proteins in acetone-fixed transfected cells by IFA (Figure 5.4).

Table 5.3. Reactivity of anti-CHIKV mAbs against full-length, C- or N-terminally truncated versions of rCap in Western blot.

Monoclonal Antibody	Group	Reactivity in Western blot												
		FL	N1	N2	N3	N4	N5	N6	C1	C2	C3	C4	C5	C6
1.7B2	1	+	+	+	+	+	-	-	-	-	-	-	-	-
4.1H11	1	+	+	+	+	+	-	-	-	-	-	-	-	-
5.2H7	1	+	+	+	+	+	-	-	-	-	-	-	-	-
5.5D11	1	+	+	+	+	+	-	-	-	-	-	-	-	-
5.5G9	1	+	+	+	+	+	-	-	-	-	-	-	-	-
5.1B12	2	+	-	-	-	-	-	-	-	-	-	-	-	-
5.5A11	2	+	-	-	-	-	-	-	-	-	-	-	-	-
4.8E2	2	+	-	-	-	-	-	-	-	-	-	-	-	-
4.10A11	2	+	-	-	-	-	-	-	-	-	-	-	-	-
5.2F8	2	+	-	-	-	-	-	-	-	-	-	-	-	-
5.4G8	2	+	-	-	-	-	-	-	-	-	-	-	-	-

When all mAbs were tested against C-terminally truncated forms of CP, none of the antibodies from either group recognized any of the truncated proteins in IFA (Figure 5.4) or Western blot (Table 5.3) despite their recognition by the anti-V5 antibody, suggesting that the C-terminal region of CP might be required for authentic antigenic structure of CP. This was confirmed by the reactivity of mouse polyclonal anti-CHIKV antibody with rCap and N-terminally truncated proteins (N1-4) in Western blot but none of the C-terminally truncated proteins (results not shown).

5.4.3. Group 1 mAbs bind native N-terminally truncated CP in CHIKV lysates

IFA and Western blot analysis of N-terminally truncated recombinant CP indicated that group 1 mAbs recognized a region in the C-terminal half of the CHIKV CP while group 2 mAbs bound the N-terminal region. We also observed that, in addition to detecting the rCap in lysates of CHIKV-infected cells (~35 kDa), group 1 mAbs also detected smaller, truncated versions of native CP (sCP) ranging from ~15-30 kDa (Figure 5.6A). In contrast, antibodies from group 2 only recognized full-length CP in these lysates (Figure 5.6B).

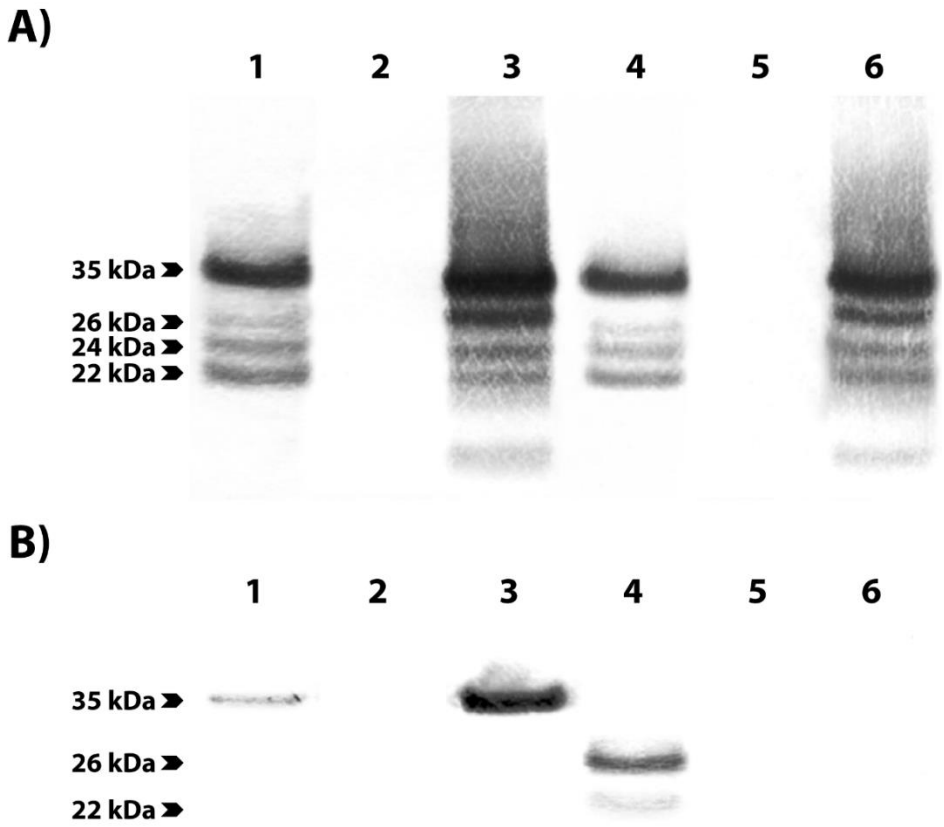
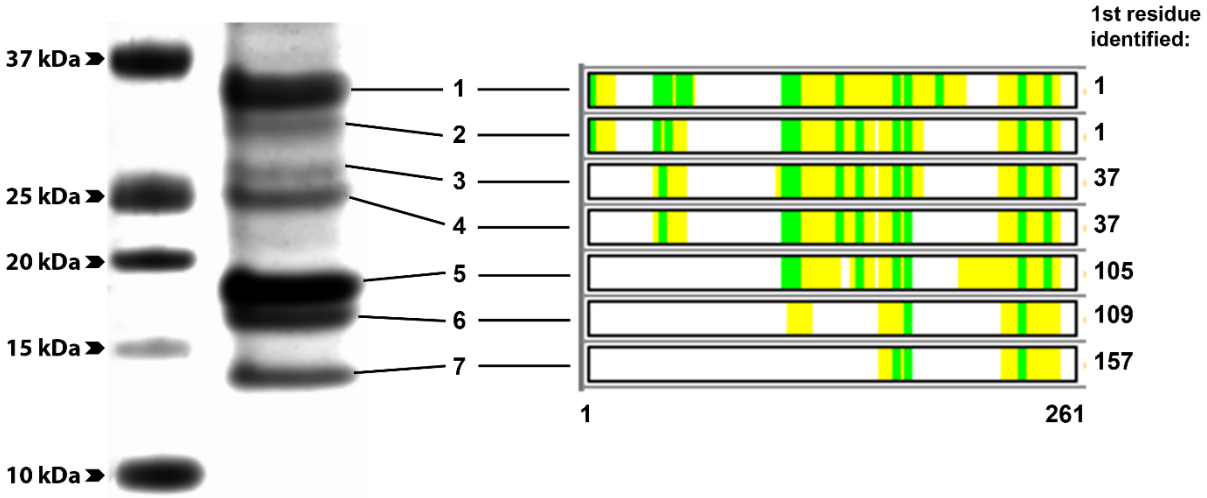


Figure 5.6. Reactivity of anti-CP mAbs with full length and truncated versions of native and recombinant CP. Reactivity of mAbs 1.7B2 (lanes A1-3), 5.2H7 (lanes A4-6), cocktail of group 2 mAbs (lanes B1-3) and anti-V5 mAb (lanes B4-6) in Western blot against boiled and reduced lysates of COS-7L cells expressing rCap (lanes 1 & 4), mock-transfected COS-7L cells (lanes 2 & 5), and CHIKV-infected C6/36 cells (lanes 3 & 6).

To determine the identity of the truncated native versions of CP, capsid proteins were immune-precipitated from CHIKV-infected lysates with protein G beads coupled to mAb 1.7B2 (group 1). Resulting pull-downs were resolved on SDS-PAGE and selected Coomassie-stained CPs were analysed by mass spectrometry. These analyses showed that all species of CP recognized by mAb 1.7B2, including the rCap, contained the same C-terminal peptide (Figure 5.7). However, as the CP molecules were truncated, detection of the N-terminus region was progressively lesser, with observations that peptides toward the N-terminal side of the protein, that were previously identified in larger-sized bands, were missing. No smaller molecules of CP were precipitated, which was consistent with the binding pattern of 1.7B2 and other group 1 mAbs to N-terminally-truncated recombinant CPs; binding to N1 to N4 (35 to 140 residues removed) but not N5 and N6 (175 and 210 residues removed). Together, these data confirm that the epitopes recognized by 1.7B2, and other group 1 mAbs, were located between residues 140 and 210 in the C-terminal half of CP. Furthermore, the lack of recognition of native CP

truncated by 36 or more residues from the N-terminus also supports their binding to an N-terminal domain - within the first 35 aa - of the protein.



1)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQR**QAGQLAQLISAVNKLTMR**AVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRRER**MCMKIENDCIFEVKHEGKVTGYACLVGDKVMKPAHVKGTIDNADLAK**LAFK**RSSKYDLECAQIPVHMKSD**
ASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNK**GRVVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

2)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQR**QAGQLAQLISAVNK**LTMRAVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRRER**MCMKIENDCIFEVKHEGKVTGYACLVGDKVMKPAHVKGTIDNADLAK**LAFKR**SSKYDLECAQIPVHMKSD**
ASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNK**GRVVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

3)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQR**QAGQLAQLISAVNK**LTMRAVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRR**ERMCMKIENDCIFEVKHEGKVTGYACLVGDKVMKPAHVKGTIDNADLAK**LAFKR**SSKYDLECAQIPVHMKSD**
ASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNK**GRVVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

4)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQR**QAGQLAQLISAVNK**LTMRAVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRRER**MCMKIENDCIFEVK**HEGK**VTGYACLVGDKVMKPAHVKGTIDNADLAK**LAFKR**SSKYDLECAQIPVHMK**SD
ASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNK**GRVVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

5)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQRQAGQLAQLISAVNKLTMRAVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRRER**MCMKIENDCIFEVK**HEGK**VTGYACLVGDK**VMKPAHVK**GTIDNADLAK**LAFKR**SSKYDLECAQIPVHMK**SD
ASKFTHEKPEGYYNWHHGAVQYSGGR**FTIPTGAGKPGDSGRPIFDNKGRVVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

6)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQRQAGQLAQLISAVNKLTMRAVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRRERMCMK**IENDCIFEVK**HEGKVTGYACLVGDKVMKPAHVKGTIDNADLAKLAFKR**SSKYDLECAQIPVHMK**SD
ASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGR**VVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

7)
MEFIPTQTFYNRRYQPRPWTPRPTIQVIRPRPRPQRQAGQLAQLISAVNKLTMRAVPQQKPRRNRKNKKQKQKQQAPQNNTNQKKQP
PKKKPAQKKKKPGRRERMCMKIENDCIFEVKHEGKVTGYACLVGDKVMKPAHVKGTIDNADLAKLAFKR**SSKYDLECAQIPVHMK**SD
ASKFTHEKPEGYYNWHHGAVQYSGGRFTIPTGAGKPGDSGRPIFDNKGR**VVAIVLGGANEGARTALSVVTWNKDIVTK**ITPEGAEEW

Figure 5.7. Coverage of the CHIKV capsid protein by peptides identified via mass spectrometry analysis of different bands excised from SDS-PAGE of boiled and reduced proteins immunoprecipitated by a group 1 mAb, 1.7B2. Sequences for peptides identified within each respective CHIKV CP band (1-7) are bolded and highlighted in yellow.

5.4.4. Group 1 mAbs target a series of overlapping epitopes on CP

To further define the binding sites of the mAbs from group 1, and determine the topology of their epitopes in the C-terminal half of CP, representative group 1 antibodies were tested in a competitive binding assay against one another using ELISA. Three of the five mAbs in this group (1.7B2, 4.1H11 and 5.2H7) were successfully biotinylated and subsequently assessed for competition against saturating concentrations of each of the five unlabelled mAbs to establish the degree of inhibition exhibited for each pairing. MAbs 1.7B2 and 4.1H11 showed complete two-way inhibition with each other indicating they bind to the same or highly adjacent epitopes (Figure 5.8). Partial two-way inhibition of both these mAbs with mAb 5.2H7 was also observed, suggesting that the epitope recognized by 5.2H7 is slightly different to the former pair but in the same spatial domain. The partial one-way inhibition of 5.2H7 by the unlabelled mAbs 5.5D11 and 5.5G9, but not by 1.7B2 and 4.1H11, suggests a continuum of overlapping epitopes (Figure 5.9). The competitive binding result also confirms that these antibodies bind to the same region of the CP.

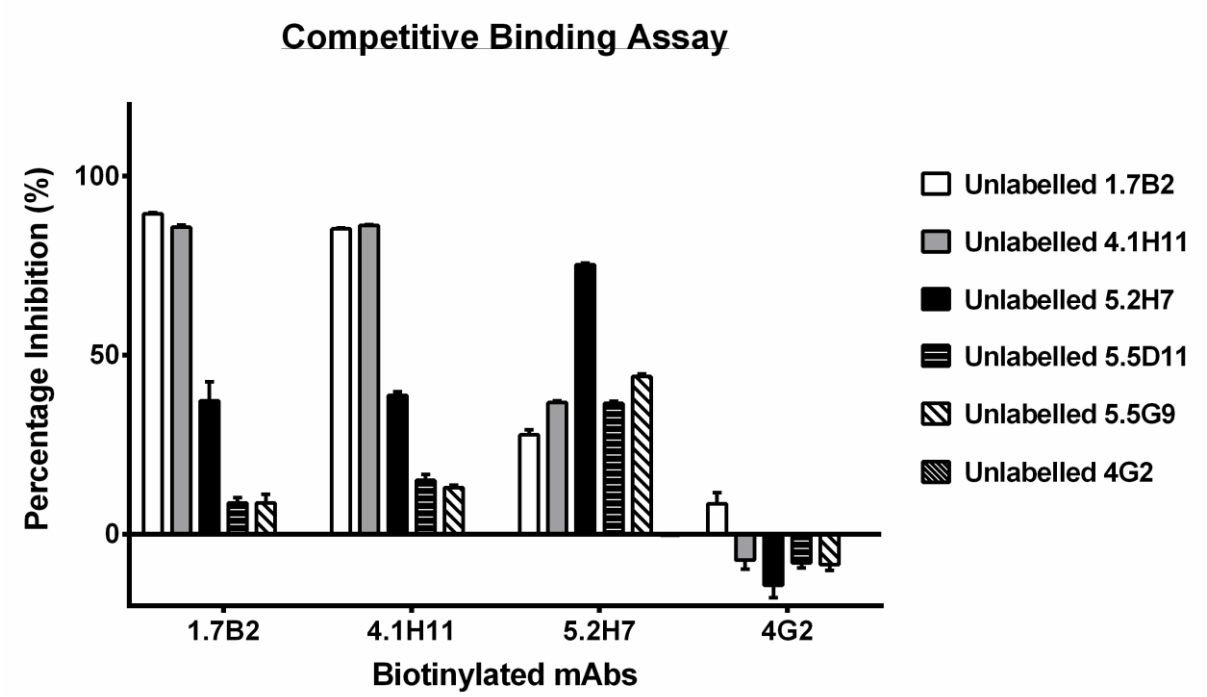


Figure 5.8. Competitive binding profiles of CHIKV CP-specific mAbs in ELISA. Antigens in lysates of $CHIKV_{MAU}$-infected Vero cells were adsorbed to 96-well plates at a 1/500 dilution prior to incubation with a saturating dilution of purified, unlabelled anti-CP mAbs. Without washing, non-saturating dilutions of biotinylated mAbs were then added as 'competitor' antibodies to respective wells. The mean absorbance reading (OD_{405nm}) of four replicates were plotted with bars showing standard error of mean (SEM). 4G2 is a control mAb

specific to the E protein of flaviviruses. Assay was optimized to obtain complete inhibition of each biotinylated mAb by its homologous unlabelled competitor.

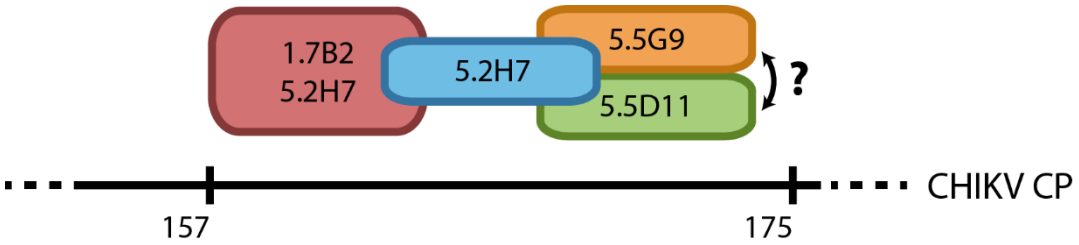


Figure 5.9. Schematic representation of proposed spatial relationship of epitopes recognized by group 1 anti-CP mAbs. Antibodies in the same coloured circle illustrates two-way inhibition, with overlaps representing one-way inhibition amongst mAbs tested. While it is safe to assume that the sequence of overlapping should be fairly accurate since the group 1 mAbs do recognize linear epitopes, the order of epitopes might be inversed. Furthermore, it is highly likely that mAbs bind from residues 157 to 175, however, a more conservative estimate would be from position 140-210.

5.5. DISCUSSION

In this study, we used a series of truncated recombinant proteins to map the binding sites of a panel of CHIKV CP-specific mAbs produced in a previous study – see Chapter 4 (Goh *et al.*, 2014). The binding patterns of the mAbs in Western blot and IFA allowed us to deduce that these antibodies bound two regions of CP; one domain was located within the first 35 aa of the N-terminus of CP (group 2 mAbs), and a second domain in the C-terminal half of the protein between residues 140 and 210 (group 1 mAbs). This was supported by mass spectrometry analysis of naturally-truncated native CP species in CHIKV-infected cell lysates that were recognized by group 1 but not group 2 mAbs, which confirmed the location of the two binding domains of these mAbs. The binding of all group 1 mAbs to a defined region between residues 140 and 210 in the C terminal half of CP was further supported by the competitive binding experiments. While the data demonstrated that these five mAbs recognized at least four different epitopes, there was significant overlap suggesting a continuum of binding sites in a defined region. The proposed spatial relationship of epitopes recognized by the group 1 mAbs is schematically presented in Figure 5.9.

Truncated fragments of CHIKV CP displaying sizes similar to those observed in this study have been previously been identified in cells expressing recombinant CHIKV CP from a baculovirus vector, as well as cells infected with a recombinant measles vaccine expressing

CHIKV virus-like particles (Cho *et al.*, 2008; Brandler *et al.*, 2013). This uncharacterized minor product of CP has also been documented in other alphaviruses such as WEEV and SINV (Ishida and Simizu, 1981; Bell *et al.*, 1983; Choi *et al.*, 1996). Based on the observations made and data obtained in this study, a schematic of the different sized CP bands derived from molecular weight analysis of both CP from crude lysates of CHIKV-infected C6/36 cells and recombinantly expressed versions, was constructed (Figure 5.10). This suggests that sCP is approximately 10-11 kDa smaller than its full-sized counterpart and is truncated by ~100 residues from the N-terminus. This was confirmed by successful retention of the C-terminally inserted V5-His tag and the mass spectrometry analysis that the N-terminal peptide identified in the major native species sCP in infected cells lysates began with the methionine at position 105 in CHIKV CP. This also corresponds with a predicted alternate translation initiation site at the third AUG of the 26S RNA in the alphavirus structural polyprotein, previously observed in a SINV mutant (Ventoso *et al.*, 2006). Irrespective of whether this minor form of CP is generated by alternate translation initiation, or protease cleavage, the fact that this truncation has been consistently detected, along with the synthesis of full-sized CP, in naturally-infected vertebrate and mosquito cells implies that sCP may have functional significance.

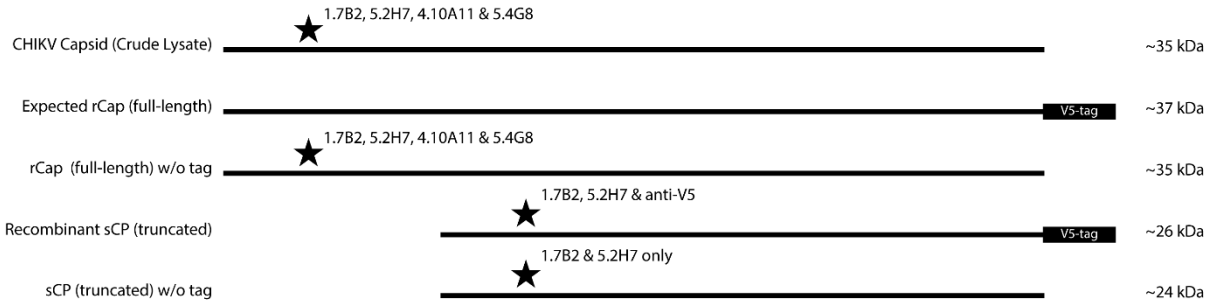


Figure 5.10. Schematic representation of different sizes of CP observed in Western blot. Stars represents the mAbs that are reactive toward that particular protein band. To obtain sizes of protein bands, a calibration curve was constructed by semi-logarithmically plotting the relative mobility values of the molecular weight standards against their known molecular weights. The relative mobility of each target protein was then utilized to estimate their molecular weight.

Our initial findings that all the anti-CP mAbs included in this study recognized reduced and carboxymethylated antigens in Western blot, indicated they bound to linear or continuous epitopes independent of tertiary structure. Thus, it was surprising that attempts to further define the binding sites of these antibodies using a series of 20-mer synthetic peptides with 10-mer overlaps covering the entire CHIKV CP were unsuccessful. None of the mAbs showed any reactivity towards the peptides when adsorbed to the solid phase in ELISA, bound onto

nitrocellulose membranes in dot blot, or when presented on microspheres in a Luminex-based assay. The successful recognition of control peptides synthesized in the same batch by their corresponding mAbs in both assay formats indicated that incorrect synthesis was not likely to be the problem. This suggested that the epitopes on CHIKV CP required additional structures for mAb recognition, potentially post-translational modifications such as phosphorylation or glycosylation, as described in previous studies (Glenney *et al.*, 1988; Kehoe *et al.*, 2006). The CP of alphaviruses are known not to harbour any N-linked glycosylation, which was previously confirmed by endoglycosidase digestion – see Chapter 4 (Goh *et al.*, 2014). However, eight potential sites of phosphorylation have been predicted in CHIKV CP based on its amino acid sequence, with two potentially phosphorylated residues at positions 8 and 20, both in the proposed binding region of group 2 mAbs, and one at residue 158 in the putative binding domain of group 1 mAbs (Figure 5.3).

The lack of reaction of all mAbs and polyclonal antisera to C-terminally truncated recombinant CP, despite their clear detection by anti-V5 in Western bot and IFA, was also unexpected considering the apparent linear nature of their epitopes. This suggests that the C-terminal residues nay be crucial to the antigenic structure of CHIKV CP, either by facilitating phosphorylation of residues in the binding sites as referred to above, or by stabilizing the overall structure of CP and exposing these epitopes on the surface of the protein, allowing access for antibody binding. Both of these hypotheses fit well with the data obtained in this study, including the failure of the antibodies to recognize the synthetic peptides; the 20-mer peptides predicted to contain the putative binding sites (peptides no. 1-4 for group 2 mAbs; no. 15-18 for group 1 mAbs) would also lack the C-terminal peptides of CP. Future studies to resolve this question and further define the binding sites of the anti-CP mAbs, could include the re-construction of rCap with variable internal deletions and the retention of the C-terminal residues.

5.6. CONCLUSIONS

In summary, we have mapped the binding sites of a panel of anti-CHIKV CP mAbs to two putative domains. Group 1 mAbs recognized an epitope(s) between residues 1-35, while group 2 mAbs bound a region between residues 140-210; the latter targeting a series of overlapping epitopes. We also provided evidence that the C-terminus of CP is required for the authentic antigenic structure of the protein - and thus antibody binding - and have identified a smaller

species of CP in CHIKV-infected cells that may represent an alternative translation product of the viral 26S RNA. Our epitope mapping studies have identified novel structural properties of the CHIKV CP and characterized useful reagents for their further investigation. A better understanding of the structure of CP will provide valuable insight into the multifunctional role of this protein in CHIKV replication, as well as that of numerous other alphaviruses.

5.7. ACKNOWLEDGEMENTS

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CHAPTER 6: USING MASS SPECTROMETRY-GUIDED SPECIFIC PRIMER DESIGN FOR GENERATION OF CHIMERIC MOUSE-HUMAN ANTIBODIES FOR CHIKUNGUNYA DIAGNOSTICS

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6.1. ABSTRACT

Chikungunya virus (CHIKV) is an arbovirus that causes febrile illness with an incapacitating and prolonged arthralgic syndrome, and has recently caused a large epidemic involving millions of infections in over 40 countries. Several ELISA-based IgM/IgG diagnostic tests have been developed for the diagnosis of CHIKV infection. However, international standardization and validation of these assays are currently limited. Presently, the use of human sera collected from infected or uninfected individuals is required for the validation of diagnostic assays, and to determine cut-off values. Numerous drawbacks are associated with the routine usage of pooled reference sera from CHIKV-infected patients, including continuity of supply, ethical and safety concerns, and batch-to-batch variation. Herein we report the generation of a genetically engineered chimeric IgG, consisting of mouse hybridoma-derived variable chain regions and human constant regions. We also describe a novel approach utilising mass spectrometry and 5′ rapid amplification of cDNA ends (5′ RACE) to design gene-specific primers, whilst avoiding the amplification of aberrant immunoglobulin genes from the parent myeloma cell lines. The resulting recombinant, chimeric antibody retained the antigen specificity of the parent hybridoma antibody.

6.2. INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-transmitted arthritogenic alphavirus from the family Togaviridae, that includes other medically significant viruses such as Ross River virus, Barmah Forest virus, o'nyong-nyong virus, Mayaro virus and Sindbis virus (Suhrbier *et al.*,

2012). Chikungunya disease in humans is characterized by acute and chronic polyarthralgia/polyarthritis (Hoarau *et al.*, 2010; Suhrbier *et al.*, 2012). The acute phase of disease is often also associated with an abrupt onset of fever, myalgia, and a rash that is usually maculopapular in appearance (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Suhrbier *et al.*, 2012).

CHIKV was first isolated in 1952 in Tanzania and has caused sporadic epidemics of primarily rheumatic disease mainly in Africa and Asia (Suhrbier *et al.*, 2012). In 2004-2011, CHIKV was responsible for up to 6.5 million infections reported in over 40 countries (Munasinghe *et al.*, 1966; Lam *et al.*, 2001; Renault *et al.*, 2007; Rezza *et al.*, 2007; Grandadam *et al.*, 2011; Suhrbier *et al.*, 2012; Horwood *et al.*, 2013; Van Bortel *et al.*, 2014). The recent epidemic has seen CHIKV associated with severe disease manifestations and mortality, primarily in elderly patients with co-morbidities, and in the young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010). Mother-to-child transmission was also observed, with approximately half the infected neonates developing serious forms of CHIKV disease characterized by one or more of the following signs: haemorrhage, disseminated intravascular coagulation, cardiac and/or neurological manifestations; the latter often leading to permanent disabilities (Lenglet *et al.*, 2006; Gerardin *et al.*, 2008; Suhrbier *et al.*, 2012). Treatment of CHIKV rheumatic disease currently involves the use of pain killers and/or non-steroidal anti-inflammatory drugs, with relief often inadequate. There is currently no licensed human vaccine available for CHIKV.

The virus co-circulates with the causative agents of several other tropical diseases such as dengue fever and typhus. Although there are no specific antivirals or vaccines available for chikungunya, timely and accurate diagnosis of CHIKV is important. Undiagnosed patients presenting non-specific fever are commonly given antimicrobials empirically and unnecessarily (Joshi *et al.*, 2008). This is a waste of resources which may contribute to antimicrobial resistance, preventable with a simple diagnostic test. Early diagnosis of CHIKV can also result in better interventions for vector control and disease management, in order to prevent further spread. Effective surveillance of emerging diseases, such as CHIKV, is crucial for alerting the local and global community to a potential threat.

Serodiagnosis by detection of IgM and IgG via ELISA are used as a standard for laboratory-based diagnoses of arthritogenic alphavirus diseases. “In-house” CHIKV ELISAs have been widely used in a number of countries due to their lower cost, rapidity and simplicity (Suhrbier

et al., 2012). However, kits utilized for the detection of antibodies against infectious agents commonly use human reference sera to establish cut-off values, concomitantly confirming the integrity of the tests (Jacobson, 1998). These sera, collected and pooled from infected individuals, are often included in commercial kits as positive controls and as cut-off calibrators. The routine use of these materials has several disadvantages. Firstly, a reliable supply of high-titre serum from confirmed cases of CHIKV infections can be difficult to obtain. In addition, ethical problems may arise from the collection of blood from individuals who are unwell, from children, or in societies where acquiring blood is prohibited. Furthermore, there are safety concerns dealing with potentially infectious human sera, from both the manufacturer and consumer perspectives. Lastly, each finite batch of collected serum will require standardization due to the batch variation of antibody titre (Hackett *et al.*, 1998). As an alternative, the generation of a non-serum-derived, recombinant positive control antibody that interacts specifically with a CHIKV protein and is recognized by an enzyme-conjugated anti-human antibody can be a solution to overcoming the disadvantages. Genetically engineered chimeric immunoglobulins consisting of mouse-derived variable chain regions and human antibody constant regions obtained from peripheral blood lymphocytes (PBL) satisfy these requirements. Chimeric antibodies have been previously shown to be useful controls in ELISA for the parasitic protozoan *Toxoplasma gondii* and the bacterium *Orientia tsutsugamushi*, responsible for scrub typhus (Hackett *et al.*, 1998; Jones and Barnard, 2007).

Primer sets are available for amplifying variable region genes from mouse hybridomas (Morrison, 2002), but these primers are not exhaustive and some hybridoma antibody genes can prove to be difficult to amplify (Carroll *et al.*, 1988; Ding *et al.*, 2010). In this study, we describe a novel method for generating the variable light chain (V_L) region derived from an anti-CHIKV monoclonal antibody (mAb), 5.2H7. The method was based on designing specific primers complementary to nucleic acid sequences encoding the light chains of the target mAb, guided by mass spectrometry, combined with the 5′ rapid amplification of cDNA ends (5′ RACE). This method should facilitate the avoidance of a common problem regarding the amplification of an aberrant kappa chain variant encoded or expressed within certain commonly employed hybridoma cell lines. After isolation of the heavy and light chain variable regions, a splice-overlap extension PCR was performed to fuse the mouse variable regions of 5.2H7 with the required human constant domains. Successfully fused genes were cloned and expressed in the mammalian cell line, COS-7L, then tested by ELISA.

6.3. MATERIALS AND METHODS

6.3.1. Cell lines and virus culture

C6/36 (*Aedes albopictus* mosquito) and COS-7L (African green monkey kidney) cell lines were propagated in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) during proliferation and 2% FBS for maintenance. Cultures were passaged by dissociating the cell surface monolayer from the flask with trypsin/PBS or trypsin/EDTA. C6/36 cells were incubated at 28 °C, and COS-7L cells at 37 °C, both in an atmosphere of 5% CO_2. Hybridoma cell line 5.2H7, secreting mAbs against the CHIKV capsid protein (Goh *et al.*, in press), was expanded in Hybridoma SFM (Gibco, Life Technologies) with 20% FBS at 37 °C with 5% CO_2, before being weaned off all FBS for the harvesting of mAbs from culture supernatant. All cell cultures were supplemented with 50 U penicillin mL^{-1}, 50 µg streptomycin mL^{-1} and 2 mM L-Glutamine (Gibco, Life Technologies). CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186) was used to infect C6/36 cells for crude lysate preparation. Cell monolayers were infected with CHIKV at an M.O.I. of 0.1 and incubated for 3 days prior to the removal of culture supernatant and rinsing in PBS. Cells were then disrupted by sonication in the presence of lysis buffer (BS9 containing 1% Triton X-100 and 0.1% SDS) and the resulting lysate was clarified by centrifugation at 12,000 x g for 10 min at 4 °C and stored at -20 °C until use.

6.3.2. RNA isolation and cDNA synthesis

Total RNA was isolated from 5.2H7 hybridoma cells and the myeloma parent cell line P3X63Ag8.653 using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Primer JH_R was used to synthesize the V_H chain cDNA, while the gene-specific 5.2H7_MS1-4_R primers were utilized to generate the V_L chain cDNA after 5′ RACE (refer to section 6.3.8.).

6.3.3. Generation of recombinant 5.2H7 heavy chain construct

PCR amplification of the V_H gene from 5.2H7 cells was performed by pairing a set of degenerate forward primers (Morrison, 2002), including the MVH_F primer (Table 6.1) that gave a product, with the JH_R primer. The PCR reaction consisted of 1 U Phusion polymerase (Finnzymes, Thermo Fisher Scientific), 1x Phusion HF buffer, 2 mM $MgCl_2$, 0.4 mM dNTP, 0.4 µM of each primer and approximately 100 ng of cDNA. PCR cycling conditions were as follows: initial denaturation for 30 sec (98 °C), 35 cycles of 10 sec denaturation (98 °C), 30 sec annealing (65 °C) and 10 sec extension (72 °C) and a final extension of 5 min (72 °C).

Sizes of PCR products were verified by gel electrophoresis in a Tris-borate-EDTA, pH 8.3, 1% (w/v) agarose gel stained with Red Safe Nucleic Acid Staining Solution (ABC Scientific). Purified products were sub-cloned into pGEM-T Easy vector (Promega). Similarly, genes of the human IgG heavy chain constant (C_H) region were amplified from cDNA generated from human peripheral blood leukocytes (PBL). Primer pair JH_F and CHG_R were utilized, with annealing at 60°C for 30 sec and extension at 72 °C for 30 sec. PCR products derived from the V_H and C_H region coding sequences were re-amplified from their respective pGEM-T Easy plasmids, electrophoresed and gel-extracted. The chimerization of the respective fragments was performed using a splice-overlap extension PCR (SOE-PCR) method as previously described (Jones and Barnard, 2005). Primers JH_R and JH_F are reverse complementary, therefore, equimolar amounts of mouse V_H PCR product and C_H region PCR product will hybridize and extend to produce a full length chimeric conditions in a reaction with 1 U Phusion polymerase (Finnzymes, Thermo Fisher Scientific), 1x Phusion HF buffer, 1 mM $MgCl_2$, 0.25 mM dNTP, with an initial denaturation for 3 min (98 °C), followed by 10 cycles of denaturing for 10 sec (98 °C), annealing and extension for 1 min (72 °C). Following that, the chimeric product was amplified by a subsequent PCR including the external primers (0.5 µM each of MVH_F and CHG_R) with annealing at 60°C for 30 sec and extension at 72 °C for 40 sec. The successfully chimerized product was then sub-cloned as previously described and sequenced by the Australian Genome Research Facility. After sequence confirmation, the chimeric gene was re-amplified using primers 5.2H7_V_F_NotI and CHG_R_XhoI to incorporate restriction enzyme sites, and cloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen).

Table 6.1. Nucleotide sequences of primers for generation of chimeric 5.2H7 mAb.

Primer Name	Primer Code	Type/Species	Sequence (5′ to 3′)	Tm (°C)
5.2H7 Mass Spec Frag 1 Reverse	5.2H7_MS1_R	Mouse	**RAANACRTARTGNGTRTTRAA**	65.5
5.2H7 Mass Spec Frag 2 Reverse	5.2H7_MS2_R	Mouse	**NGTRTCNACNCCYTGNGT**	47.9
5.2H7 Mass Spec Frag 3 Reverse	5.2H7_MS3_R	Mouse	**CCARTTNGCRTARTTRTG**	55.1
5.2H7 Mass Spec Frag 4 Reverse	5.2H7_MS4_R	Mouse	**NCCNCCNCCRAANTGRAA**	47.1
5.2H7 Light Leader Seq Forward	5.2H7_LS_F	Mouse	CCACC**ATG**GCCTGGACTTCACTTATACTCTCT	56.2
5.2H7 Light Leader Seq Forward with HindIII	5.2H7_LS_F_HindIII	Mouse	ATATAT**AAGCTT**CCACC**ATG**GCCTGGACTTCACTTATACTCTCT	63.4
Variable Heavy Forward [#]	MVH_F	Mouse	**CCACCATGGRATGSAGCTGKGTMATSCTCTT**	64.2
Variable Kappa Forward [#]	MVK_F	Mouse	**CCACCATGGAGACAGACACACTCCTGCTAT**	
5.2H7 Heavy Variable Forward with NotI	5.2H7_V_F_NotI	Mouse	ATATAT**GCGGCCGC**TCCACCAT**GGAATGGAGCTGGGTCTTTCTCTTC**	70.2
J-Light (Lambda) Forward	JL_F	Human/Mouse	**GGVACCAAGSTSACYGTCCTA**	59.2
J-Light (Lambda) Reverse	JL_R	Human/Mouse	**TAGGACRGTSASCTTGGTBCC**	59.2
J-Heavy Forward [‡]	JH_F	Human/Mouse	**GGGGCCAAGGGACCATGGTCACCGTCTCCTCAG**	70.9
J-Heavy Reverse [‡]	JH_R	Human/Mouse	**CTGAGGAGACGGTGACCATGGTCCCTTGGCCCC**	70.9
Igλ Light Constant Reverse	CL_R	Human	**CTA**TGAACATTCTGTAGGGGCCAC	57.3
Igλ Light Constant Reverse with XhoI	CL_R_XhoI	Human	ATATAT**CTCGAG**CTATGAACATTCTGTAGGGGCCAC	62.5
IgG Heavy Constant Reverse [‡]	CHG_R	Human	**TCA**TTTACCCGGAGACAGGGAG	58.2
IgG Heavy Constant Reverse with XhoI	CHG_R_XhoI	Human	ATATAT**CTCGAG**TCATTTACCCGGAGACAGGGAG	62.2

Bolded texts represent template-binding regions; **red** texts highlight restriction enzyme sites; underlined texts indicate start or stop codon in forward or reverse primers, respectively.

[#] Primers adapted from Morrison (2002).

[‡] Primers adapted from Jones and Barnard (2005).

6.3.4. SDS-PAGE and Coomassie blue staining

Serum-free CHIKV CP-specific hybridoma 5.2H7 culture supernatant was clarified, and antibody was purified using a HiTrap Protein G HP column (GE Healthcare). The purified antibody was prepared in 1 X NuPAGE LDS sample buffer (Invitrogen) with 10 mM dithiothreitol and heated to 95 °C for 5 min. The antibody was resolved on a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen, Life Technologies) at 170 V for 30 min. Following that, the gel was incubated in Coomassie blue stain (1% (w/v) Coomassie R250, 10% glacial acetic acid, 40% methanol in ddH_2O) at room temperature (RT) for 30 min before being rinsed twice with destaining solution (10% glacial acetic acid, 40% methanol in ddH_2O). Gel was then incubated in destaining solution overnight with rocking.

6.3.5. Sample preparation for LC-MS

The band of interest, 5.2H7 mAb light chain, was excised from the SDS-PAGE gel and destained with 50 mM ammonium bicarbonate (pH 7.8)/50% (v/v) acetonitrile for 3 hr. Proteins were then subjected to reduction with 10 mM dithiothreitol at 60 °C for 30 min followed by the addition of 55 mM iodoacetamide at RT for 30 min, in the dark, to alkylate proteins. Gel slices were washed twice with 50 mM ammonium bicarbonate (pH 7.8) by vortexing for 2 min, and dehydrated with 100% acetonitrile for 5 min. Rehydration was performed by incubating gel fragments in 8 µl enzyme solution (10 ng/uL in 50 mM ammonium bicarbonate, pH 7.8, of either trypsin, chymotrypsin or AspN) at 4 °C for 20 min. To prevent drying out, 12 µl of 50 mM ammonium bicarbonate was added to gel fragments before proteins were allowed to digest overnight at 37 °C. Digested peptides were extracted from the gel by sonication three times in 50% acetonitrile/0.1% trifluoroacetic acid for 10 min. Peptide-containing supernatants were then lyophilized in a Savant SPD131DDA SpeedVac Concentrator (Thermo Scientific) at 45 °C. Samples were resuspended in 0.1% trifluoroacetic in 50% acetonitrile and cleaned up using C18 ZipTips (Merck Millipore) according to manufacturer's instructions.

6.3.6. Mass spectrometry

LC-MS/MS analysis was performed similar to that described in Kappler & Nouwens (2013), with modifications. In brief, samples were separated on a Shimadzu Prominence nanoLC system. Peptides were first desalted on an Agilent C18 trap (0.3 x 5mm, 5 µm) for 3 min, at a flow rate of 30 µL/min using buffer A, followed by separation on a Vydac Everest C18 column (300 A, 5 µm, 150 µm x 150 mm) at a flow rate of 1 µL/ min. Peptides were separated using a gradient of 10-40% buffer B over 30 min, followed by 40-98% buffer B over 3 min, where buffer A = 1% acetonitrile/0 .1 % formic acid, and buffer B = 0.1% formic acid in acetonitrile. A TripleTof 5600 (ABSciex) was used to directly analyze eluted peptides using a Nanospray III interface. A TOF MS scan was performed

across m/z 350 - 1800 for 0.5 sec, followed by data-dependent acquisition of up to 20 peptides across m/z 40 - 1800 (0.05 sec/spectrum) with intensity above 100 counts using rolling collision energy. Gas and voltage settings were adjusted as required.

6.3.7. De novo peptide sequencing

MS data was initially searched using MASCOT to filter out conserved antibody peptides (including the known myeloma-derived aberrant variable regions sequence), searching all species in the SwissProt database. Enzyme was set to trypsin, AspN or chymotrypsin, respectively, allowing for up to two mis-cleavages, using a mass tolerance of 50 ppm for MS and 0.1 Da for MS/MS. Carboxymethylation of cysteine (fixed) and oxidation of methionine (variable) were included as modifications. Unmatched spectra from MASCOT searches were then manually inspected and de novo sequenced to determine amino acid sequence. PEAKS Studio v6.0 was also used to facilitate *de novo* sequencing. *De novo* settings included 20 ppm error for MS and 0.1 Da for MS/MS, carboxymethylation of cysteine and oxidation of methionine, with up to three post-translational modifications per peptide, and five peptide sequences generated for each spectra. Computationally determined sequences were manually validated to confirm matches.

6.3.8. Generation of recombinant 5.2H7 light chain construct

Degenerate reverse primers were designed (5.2H7_MS1_R, 5.2H7_MS2_R, 5.2H7_MS3_R and 5.2H7_MS4_R), with the help of a mouse codon usage table (http://www.kazusa.or.jp/java/codon_table_java/), based on four peptides that were considered to be unique towards the 5.2H7 V_L sequence. These primers were used as gene-specific reverse primers for the 5′ Rapid Amplification of cDNA Ends (5′ RACE) system (Invitrogen), according to manufacturer's instructions, on the 5.2H7 hybridoma RNA to obtain a PCR product extending from the front of the V_L chain to the binding site of the mass-spectrometry derived reverse primers. The 5.2H7_MS1_R primer was able to efficiently amplify a distinct product and this product was subsequently cloned into a pGEM-T Easy vector (Promega) and sequenced. IMGT/V-QUEST analysis revealed that the sequence was a lambda light chain isotype, and Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP-3.0/) analysis identified the presence of a functional antibody leader sequence. A gene-specific forward primer, 5.2H7_LS_F, and a lambda J-region reverse primer (JL_R) were thus created for the amplification of the 5.2H7 V_L chain sequences. Additionally, lambda J-region forward (JL_F) and lambda light chain constant region reverse (CL_R) primers were designed with the help of IMGT's Ig repertoire (http://www.imgt.org/IMGTrepertoire/Proteins/index.php). RT-PCR was carried out, as previously described, to obtain 5.2H7 V_L and human Ig lambda C_L gene segments. PCR products then underwent

SOE-PCR as described above before being re-amplified with 5.2H7_LS1_F_HindIII and CL_R_XhoI to include RE sites. The amplified product was cloned into pcDNA3.1 (+) mammalian expression vector.

6.3.9. Recombinant mouse-human 5.2H7 mAb expression

COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. A 1:1 molar ratio of recombinant 5.2H7 heavy: light chain pcDNA3.1 (+) construct was used for the transfection. Recombinant antibody-containing culture supernatant and cell lysates were harvested 2 days post-transfection. Supernatant was clarified by centrifugation at 12,000 x g for 10 mins at 4 °C, while cell monolayers were detached using a cell scraper and subjected to sonication in PBS prior to clarification.

6.3.10. Western blot

$CHIKV_{MAU}$ antigen or recombinant mAb supernatant/cell lysate were heated in 1 X NuPAGE LDS sample buffer (Invitrogen), with or without 10 mM dithiothreitol, and resolved by SDS-PAGE as described above. Proteins were then transferred onto Hybond C nitrocellulose membranes (Amersham) and immune-stained as previously described in section 3.3.7., with slight modifications (Goh *et al.*, 2013). After blocking, membranes were probed either with the recombinant 5.2H7 mAb at a 1:20 dilution, or a cocktail of anti-human Ig Lambda light chain mouse antibody (BioLegend) at a 1:2,000 dilution and anti-human IgG heavy chain rabbit antibody (DAKO) at 1:500, against the CHIKV antigens and recombinant 5.2H7 supernatant/cell lysate, respectively, for 1 h. CHIKV lysate samples probed with the recombinant mAb were then allowed to incubate with the anti-human mAbs for an additional hour. Thereafter, HRP-conjugated goat anti-mouse IgG (DAKO) and goat anti-rabbit IgG (DAKO) were used as detecting antibodies at dilutions of 1:4,000 and 1:2,000, respectively. Finally, the blots were developed in DAB substrate solution (1.5 mM 3,3′-diaminobenzidine, 0.06% (v/v) H_2O_2 in PBS, pH 7.2) for 15 min at RT.

6.3.11. ELISA

Harvested recombinant mAbs were tested in ELISA as described previously (Hall *et al.*, 1988; Clark *et al.*, 2007). Briefly, $CHIKV_{MAU}$ lysate or uninfected C6/36 cell lysate were coated overnight onto 96-well plates in coating buffer (0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, pH 9.6) at 4 °C, prior to blocking. Samples were then probed with 5.2H7 hybridoma supernatant or recombinant mAb supernatant/cell lysate, serially diluted 2-fold down the plate. Bound chimeric antibodies were then incubated with a cocktail of anti-human mAbs exactly as described in the Western blot section above. The appropriate HRP-conjugated goat anti-mouse and/or anti-rabbit IgG (DAKO) were used as detecting antibodies at dilutions of 1:4,000 and 1:2,000, respectively. ABTS substrate solution (0.02% (w/v) 2, 2'-azino-

bis-3-ethylbenzthiazoline-6-sulphonic acid, 0.06% (w/v) H_2O_2 in 200 mM Na_2HPO_4 and 100 mM citric acid) was then added and allowed to develop for 30 min at RT in the dark prior to absorbance measurement at 405 nm optical density using a Labsystems Multiscan EX Type 355 UV plate reader (Pathtec).

6.4. RESULTS

6.4.1. Amplification of chimeric 5.2H7 heavy chain

Murine V_H and C_H region genes were successfully amplified using cDNA synthesised from extracted 5.2H7 hybridoma genomic RNA, with the primer sets MVH_F and JH_R, and JH_F and CHG_R, respectively. Agarose gel electrophoresis demonstrated that the PCR products were ~400 bp (V_H) and ~1 kb (C_H), as expected (Figure 6.1A). SOE-PCR was used to hybridize the V_H and C_H genes, as shown in Figure 6.1B. The product was approximately 1.4 kb in size, confirming the successful chimerization of the heavy chain genes.

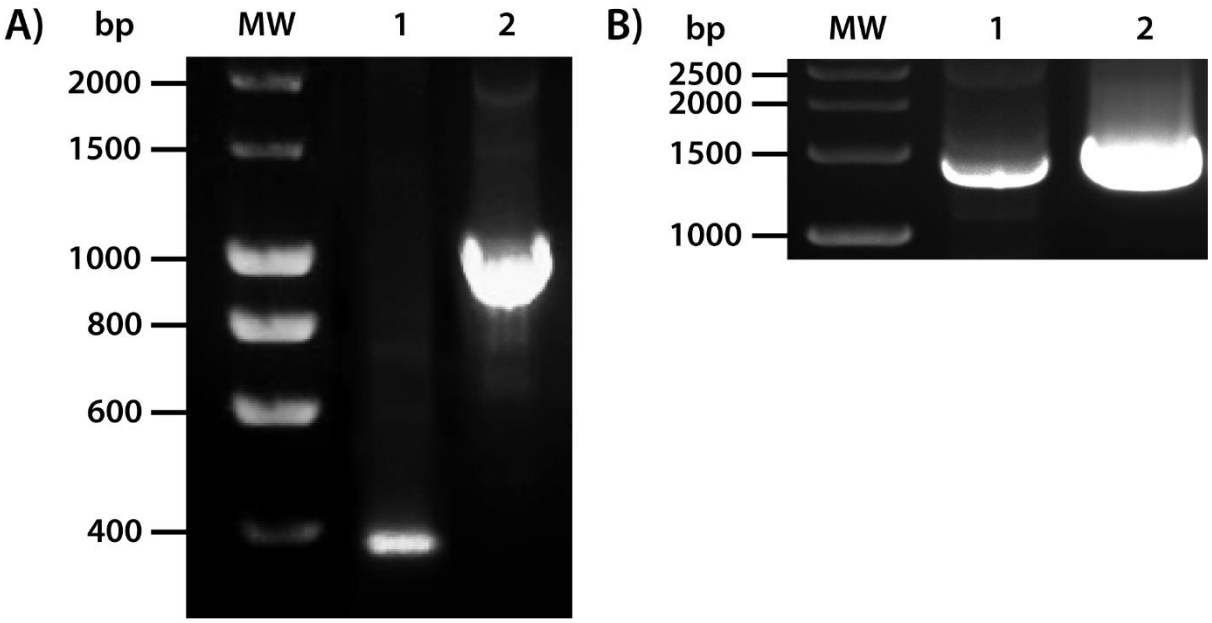

Figure 6.1. Agarose gel electrophoresis of PCR products for generation of chimeric 5.2H7 heavy chain. A) Murine V_H and human IgG C_H region were amplified from 5.2H7 hybridoma RNA and human PBL mRNA via RT-PCR. Lane MW: DNA marker; lane 1: 5.2H7 V_H PCR product; and lane 2: human IgG C_H PCR product. B) SOE-PCR was utilized to hybridize the murine V_H and human IgG C_H genes prior to re-amplification with primers containing restriction enzyme sites for cloning into pcDNA3.1 (+). Lane MW: DNA marker; lane 1: chimeric 5.2H7 heavy chain product obtained from SOE-PCR; and lane 2: re-amplified chimeric 5.2H7 heavy chain with RE sites.

6.4.2. Amplification of chimeric 5.2H7 light chain

Four mass spectrometry-derived peptides that were of unique amino acid sequences and showed no homology toward the MOPC-21 variable chain aberrant genes were used to design reverse primers to reverse-transcribe the 5.2H7 hybridoma RNA (Table 6.1). Utilizing these primers in the 5′ RACE system (Invitrogen), partial segments of the 5.2H7 V_L gene were successfully amplified, sub-cloned and sequenced. Contrary to results of the Mouse Typer Isotyping Panel kit (Bio-Rad Laboratories), IMGT/V-QUEST analysis showed that mAb 5.2H7 harbored a lambda V_L chain instead of a kappa one. Created from the 5′ sequence of the V_L chain, gene-specific primer 5.2H7_LS_F, was used in combination with JL_R to re-amplify the V_L gene, while the human lambda C_L gene was successfully generated using JL_F and CL_R. Agarose gel electrophoresis revealed that the PCR products were ~390 bp (V_L) and ~360 bp (C_L), as expected (Figure 6.2A). SOE-PCR was performed to hybridize the V_L and C_L genes, which were re-amplified prior to cloning into pcDNA3.1 (+). Figure 6.2B shows that the successfully chimerized light chain genes were approximately 700 bp in length.

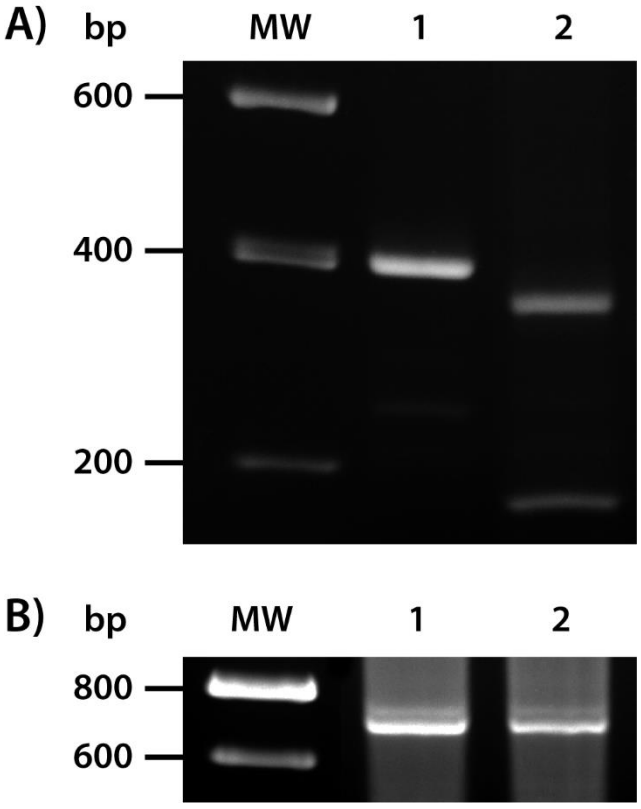

Figure 6.2. Agarose gel electrophoresis of PCR products for generation of chimeric 5.2H7 light chain. A) Murine V_L and human Ig lambda C_L region were amplified from 5.2H7 hybridoma RNA and human PBL mRNA via RT-PCR. Lane MW: DNA marker; lane 1: 5.2H7 V_L PCR product; and lane 2: human Ig lambda C_L PCR product. B) SOE-PCR was performed to hybridize the murine V_L and human Ig lambda C_L genes prior to re-amplification with primers containing RE sites for cloning into pcDNA3.1 (+). Lane MW: DNA marker; lane 1: chimeric 5.2H7 light chain SOE-PCR product; and lane 2: re-amplified chimeric 5.2H7 light chain with RE sites.

6.4.3. Chimeric mouse-human 5.2H7 detects CHIKV in multiple assays

Transfected COS-7L cells secreted fully-assembled, recombinant 5.2H7 mAb (Figure 6.3). Clarified culture supernatants of transfected cells showed strong reactivity against CHIKV lysates in Western blot (Figure 6.4), detecting a capsid protein band of ~31 kDa. Both transfected COS-7L cell lysates and supernatant were able to bind to $CHIKV_{MAU}$ antigen in ELISA format (Figure 6.5). These results confirm that the chimeric antibody retained the binding specificity of the parent hybridoma.

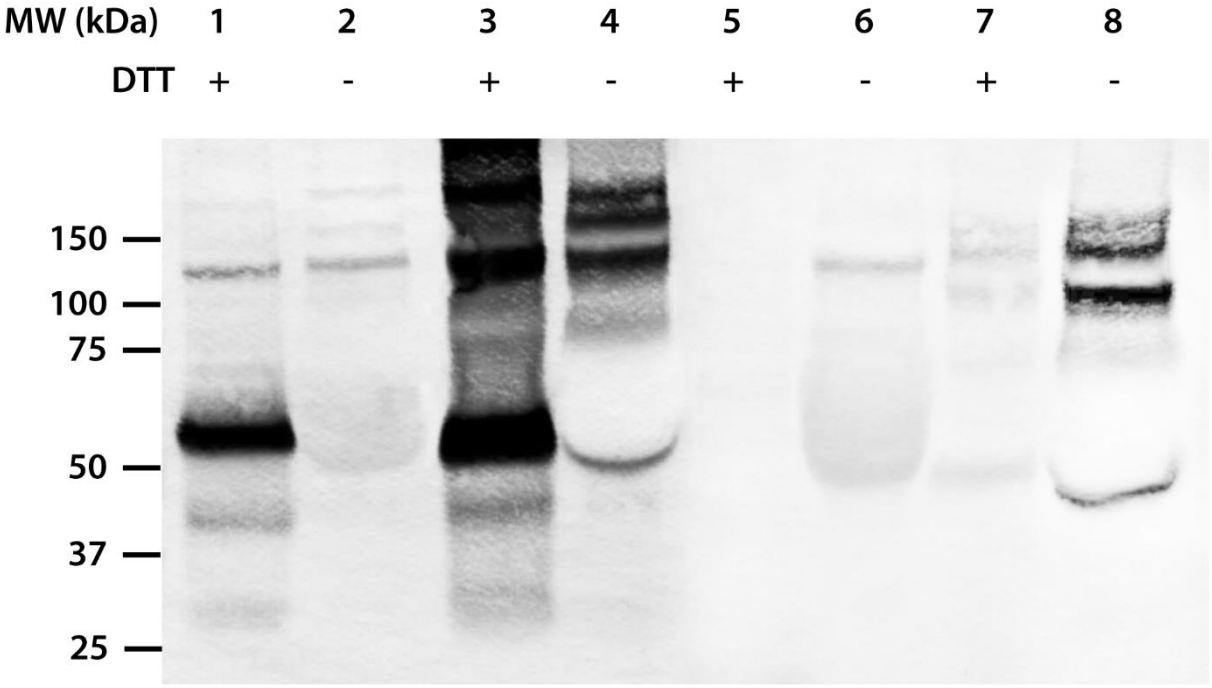

Figure 6.3. Western blot analysis of chimeric 5.2H7 expressed in COS-7L cells. Boiled, DTT reduced (+) or unreduced (-) lysates/supernatant of chimeric 5.2H7 mAb harvested from transfected COS-7L cells were probed with cocktail of anti-human mAbs prior to detection using HRP-conjugated antibodies. Lanes 1 & 2: chimeric 5.2H7 cell lysate; lanes 3 & 4: chimeric 5.2H7 supernatant; lanes 5 & 6: mock-transfected COS-7L cell lysate; and lanes 7 & 8: mock-transfected COS-7L supernatant.

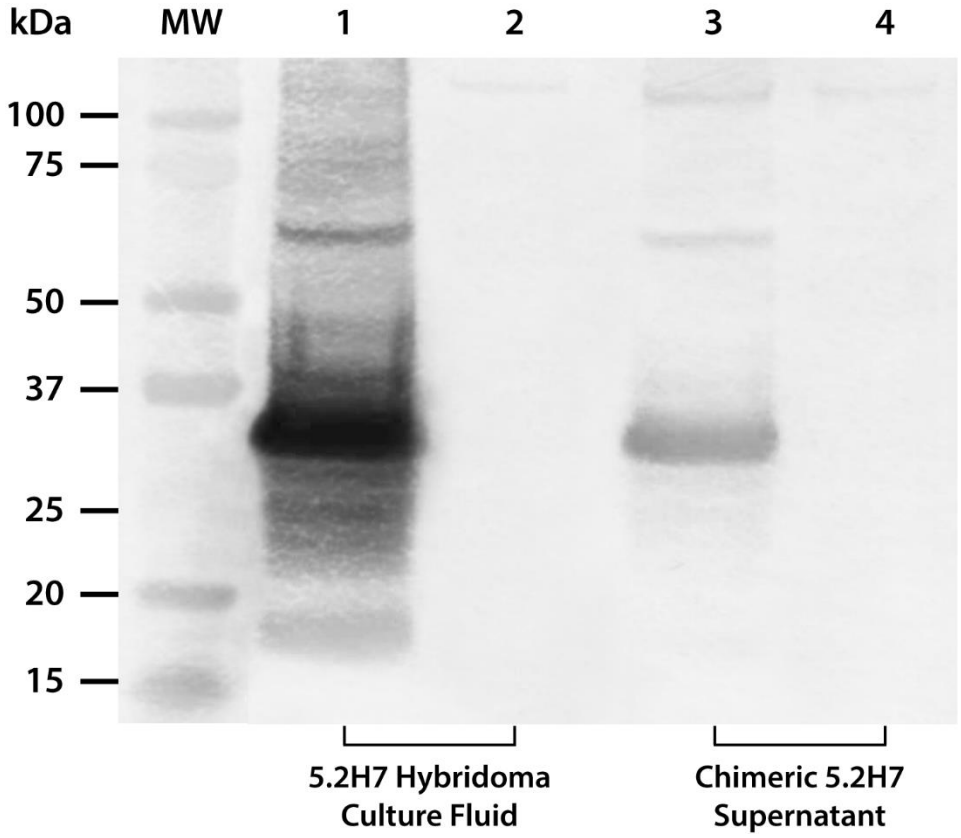

Figure 6.4. Detection of CHIKV antigen by 5.2H7 hybridoma antibody and chimeric 5.2H7 antibody by Western blot. Boiled, reduced lysates of CHIKV-infected C6/36 cells were probed either with 5.2H7 hybridoma culture supernatant or supernatant from COS7 cells transfected with chimeric 5.2H7 mAb, prior to detection with anti-mouse or anti-human secondary antibodies, respectively. Uninfected C6/36 cells were used as negative controls. Lane MW: Protein standard; lanes 1 & 3: CHIKV-infected C6/36 cell lysate; and lanes 2 & 4: uninfected C6/36 cell lysate.

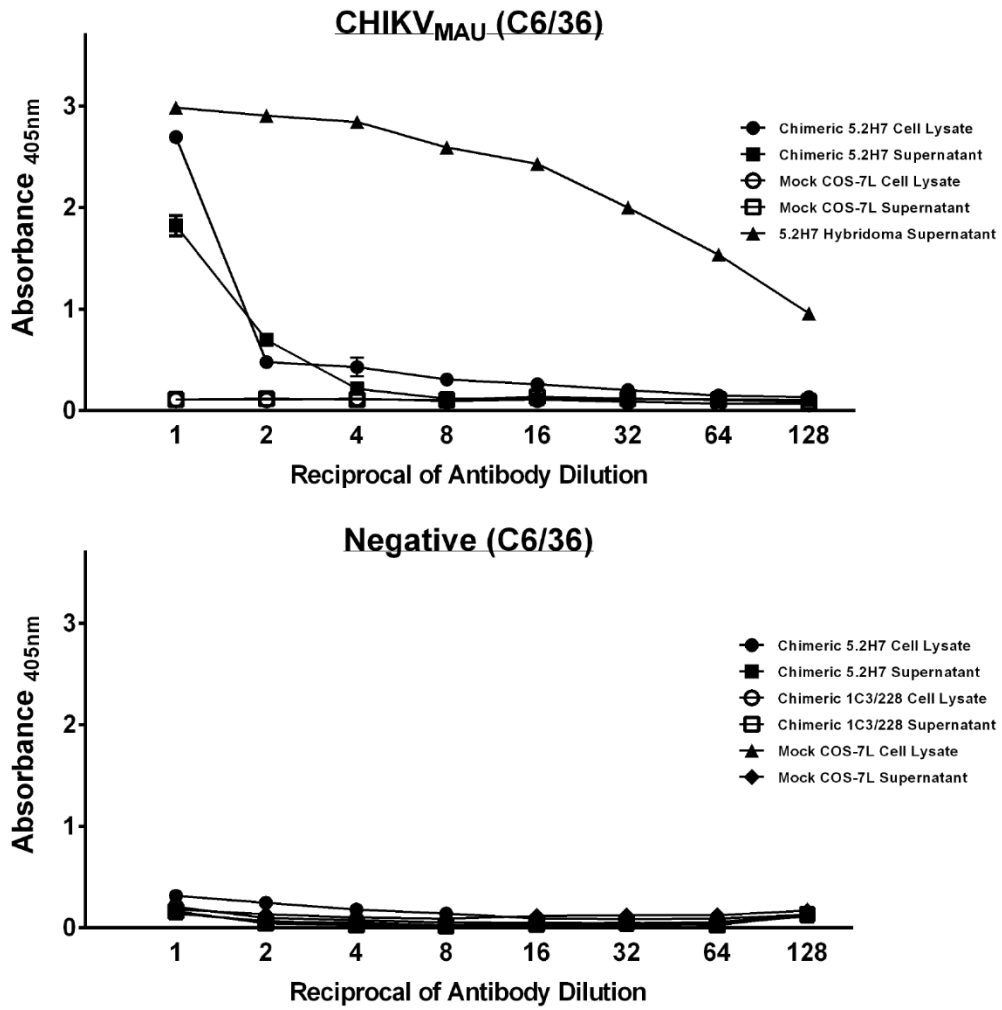

Figure 6.5. Detection of CHIKV by chimeric 5.2H7 mAb in ELISA. Crude, CHIKV-infected C6/36 cell lysates were coated onto 96-well plates prior to incubation with chimeric 5.2H7 mAb transfected COS-7L cell lysates or culture supernatant, or with 5.2H7 hybridoma supernatant. Bound antibodies were then probed with either anti-human or anti-mouse secondary antibodies, respectively, followed by detection using HRP-conjugated antibodies. The mean absorbance reading (OD_{405nm}) of duplicates from two independent experiments were plotted with error bars showing SEM. Uninfected C6/36 cell lysates were used as the negative control.

6.5. DISCUSSION

This aim of this study was to generate a fully-assembled human-mouse chimeric mAb as a positive control for use in CHIKV diagnosis. The first step in attempting to create a chimeric or humanized mAb is to clone the functional immunoglobulin variable genes via PCR amplification using degenerate primers and the cDNA of a hybridoma as template. However, the occurrence of transcribing non-functional genes from aberrant heavy and/or kappa light chain mRNA transcripts is relatively high (Carroll *et al.*, 1988; Kutemeier *et al.*, 1992; Ding *et al.*, 2010). The myeloma cell line P3X63Ag8.653 used for the creation of the 5.2H7 hybridomas was derived from MOPC-21 cells, which are known to express endogenous immunoglobulin heavy and light chain transcripts, as well

as harbor aberrant, non-functional genes (Strohal *et al.*, 1987; Irani *et al.*, 2008; Ding *et al.*, 2010). Amounts of these unproductive transcripts can be extremely high in some hybridomas and have been shown to exceed levels of their functioning counterparts (Carroll *et al.*, 1988; Irani *et al.*, 2008). The construction of chimeric mAbs using these abnormal genes may produce immunoglobulin molecules that either exhibit a significant loss in antigenic affinity, or are completely defective. Although there are strategies that can reduce the amplification of these aberrant genes, the use of variable region gene-specific primers in a high-specificity PCR should be considered as one of the most accurate methods (Duan and Pomerantz, 1994; Ostermeier and Michel, 1996). This technique can furthermore ensure that the antibody's natural leader sequence can be retained.

In this study, a degenerate primer (Morrison, 2002) was successfully used to amplify a V_H gene of mAb 5.2H7 which was determined to be functional by IMGT analysis, and was distinct from the known MOPC-21 non-functional heavy chain. However, in contrast, the use of degenerate primers to amplify the V_L gene of 5.2H7 was not successful. The Mouse Typer Isotyping Panel kit (Bio-Rad Laboratories) utilized in our study determined that the light chain of 5.2H7 was of the kappa isotype. Nonetheless, the degenerate kappa light chain-specific primers were only capable of amplifying genes of the aberrant transcript, requiring the design of a more specific primer for the 5.2H7 light chain. N-terminal sequencing was performed on the light chain of mAb 5.2H7. However, we were unable to obtain any amino acid sequences, even after several attempts of unblocking procedures, due to the presence of an unknown N-terminal modification. Mass spectrometry was therefore utilized to obtain peptide fragments of the 5.2H7 V_L region. Peptides with sequences homologous with regions of the MOPC-21 light chain (NCBI Accession No. M35669, GI: 197295) were discarded. Primers designed off the identified peptide sequences were used in conjunction with the 5′ RACE system to obtain partial 5.2H7 V_L cDNA. Following further amplification and sub-cloning, the incomplete V_L gene was sequenced and a functional leader sequence was identified, allowing a region for the design of a gene specific forward primer. The IMGT/V-QUEST analysis also revealed that the light chain of mAb 5.2H7 was of a lambda isotype, despite the results obtained with mAb isotyping kit that indicated a kappa chain. Therefore, lambda light chain J-region reverse and forward primers were designed for use in PCR amplification of the 5.2H7 V_L region and human lambda C_L region, respectively.

Although the specific amplification method presented in this study was used for the generation of a V_L gene, it can also be efficiently utilized for the generation of heavy chain genes. Few aberrant V_H genes have been reported, yet, they are known to be far more complicated and diversified than the well-recognized aberrant V_L gene. Moreover, more than one aberrant V_H transcript may exist in a single hybridoma (Irani *et al.*, 2008). Currently, the best strategy to avoid the non-functional V_H genes

is by post-PCR screening via sequencing and comparative analyses in databases (Ding *et al.*, 2010). By using the method described above for the generation of the 5.2H7 V_L genes, it will be possible to eliminate the laborious task of screening numerous cloned products for the avoidance of aberrant heavy chain genes.

We have demonstrated that culture supernatant from transiently-transfected COS-7L cells contains a functional chimeric form of 5.2H7 mAb that specifically detects the CHIKV CP antigen in Western blot and ELISA and maintains the reactivity of the parent hybridoma. This chimeric construct represents a good candidate for scale-up production by establishing stable cells lines for consistent and continuous secretion of the recombinant mAbs using well described procedures (Wong *et al.*, 1996; Jones and Barnard, 2007; By *et al.*, 2009). The recombinant 5.2H7 antibody has potential application as a positive and cut-off calibrator control reagent in serology-based diagnostic assays for CHIKV, replacing the currently used human serum-derived reagents, eliminating problems associated with supply and batch-to-batch variation.

We have previously shown that mAb 5.2H7 exhibits cross-reactivity to several other alphaviruses (e.g. Ross River virus, Sindbis virus, Semliki virus; see Chapter 4), and probably to additional alphaviruses not yet assessed, thus making it a prime candidate for use as a positive control in diagnostic tests for these viruses as well (Goh *et al.*, 2014). This antibody also performed well in IFA and recognized CHIKV CP in Western blot under reducing conditions – see Chapter 4 (Goh *et al.*, 2014). Thus, the chimeric version of 5.2H7 will potentially be a useful reference reagent in a variety of assay formats.

6.6. CONCLUSIONS

In summary, we have generated a chimeric human-mouse mAb to the CP of CHIKV that represents a candidate positive control reagent in human diagnostic assays. Additionally, we have described an efficient and simple method to specifically acquire sequences of immunoglobulin variable chain regions, while avoiding the unwanted amplification of the aberrant heavy and/or light chain RNA transcripts present in several myeloma cell lines commonly used in hybridoma fusion technology.

6.7. ACKNOWLEDGEMENTS

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CHAPTER 7: A SENSITIVE EPITOPE-BLOCKING ELISA FOR THE DETECTION OF CHIKUNGUNYA VIRUS-SPECIFIC ANTIBODIES IN PATIENTS

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7.1. ABSTRACT

Chikungunya fever (CHIKF) has re-emerged as an arboviral disease that mimics clinical symptoms of other diseases such as dengue, malaria, as well as other alphavirus-related illnesses leading to problems with definitive diagnosis of the infection. Herein we describe the development and evaluation of a sensitive epitope-blocking ELISA (EB-ELISA) capable of specifically detecting anti-chikungunya virus (CHIKV) antibodies in clinical samples. The assay uses a monoclonal antibody (mAb) that binds an epitope on the E2 protein of CHIKV and does not exhibit cross-reactivity to other related alphaviruses. We also demonstrated the use of recombinant CHIK virus-like particles (VLPs) as a safe alternative antigen to infectious virions in the assay. Based on testing of 60 serum samples from patients in the acute or convalescent phase of CHIKV infection, the EB-ELISA provided us with 100% sensitivity, and exhibited 98.5% specificity when Ross River virus (RRV)- or Barmah Forest virus (BFV)-immune serum samples were included. This assay meets the public health demands of a rapid, robust, sensitive and specific, yet simple assay for specifically diagnosing CHIK-infections in humans. Lastly, we have also, for the first time, identified a CHIKV-positive serum sample within a pool of 29 patient serum samples using non-cross reactive, CHIKV-specific mAbs in an antigen-capture assay. This double-antibody assay was then transferred on a dipstick platform for the successful detection of CHIKV proteins.

7.2. INTRODUCTION

Chikungunya virus (CHIKV), the etiological agent of Chikungunya fever (CHIKF), belongs to the family *Togaviridae* and genus *Alphavirus*. CHIKV was first isolated in 1952 during an outbreak in Tanzania, East Africa (Robinson, 1955). Since then, CHIKV has caused sporadic epidemics of rheumatic disease though its prevalence has increased dramatically in recent years (Powers and

Logue, 2007). Between 2004 and 2011, CHIKV was responsible for a massive outbreak at an estimated 1.4-6.5 million infections, with imported cases reported in over 40 countries (Suhrbier *et al.*, 2012). Geographical expansion of the *Aedes albopictus* mosquito's distribution has allowed for the first autochthonous CHIKV infections in Italy and France, in 2007 and 2010, respectively (Rezza *et al.*, 2007; Angelini *et al.*, 2008; Grandadam *et al.*, 2011). The increase in international travel has also seen the virus spread to previously unaffected countries such as the USA, the Caribbean and Australasia (Schwartz and Albert, 2010; Gibney *et al.*, 2011; Viennet *et al.*, 2013; Higgs, 2014; Mansuy *et al.*, 2014).

CHIKF is characterized by an abrupt onset of fever, headache, myalgia, fatigue, nausea, rashes (usually maculopapular) and severe polyarthralgia, which can persist from weeks to months (Robinson, 1955; Brighton *et al.*, 1983; Hoarau *et al.*, 2010). The re-emergence of CHIKV has seen it be associated with severe disease manifestations and mortality, primarily in elderly patients with co-morbidities and the very young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010). Furthermore, mother-to-child transmission was also observed with about half the infected neonates developing serious disease outcomes such as haemorrhage, disseminated intravascular coagulation and/or cardiac and neurological complications, with the latter often leading to permanent disabilities (Rampal *et al.*, 2007; Gerardin *et al.*, 2008; Suhrbier *et al.*, 2012). The treatment of rheumatic disease caused by CHIKV currently involves the use of pain killers and/or non-steroidal anti-inflammatory drugs. Presently, there are no licensed human vaccines available although CHIKV vaccines are in development (Akahata *et al.*, 2010; Wang *et al.*, 2011; Brandler *et al.*, 2013; Metz *et al.*, 2013; Powers, 2014).

The clinical profile of CHIKF is similar to that of several other infections such as dengue fever, malaria and a host of closely-related arthralgic alphaviral diseases (Carey, 1971; Sergon *et al.*, 2007; Suhrbier *et al.*, 2012; Roth *et al.*, 2014). Co-infection of CHIKV with dengue virus and other tropical arboviruses that cause non-specific symptoms similar to CHIKF have also been reported, highlighting the requirement of definitive diagnostic tests to assist clinicians with treatment options as well as to inaugurate appropriate public health measures (Khai Ming *et al.*, 1974; Vazeille *et al.*, 2010; Kumar *et al.*, 2012; Baba *et al.*, 2013).

During early stages of the infection when antibody responses have not been generated, virus culture and/or PCR-based techniques are typically utilized for the detection of CHIKV. However, blood samples must be taken during the viraemic period, which typically lasts only 5-7 days (Edwards *et al.*, 2007; Laurent *et al.*, 2007; Santhosh *et al.*, 2007). Moreover, this narrow window of opportunity for nucleic acid detection often starts prior to the onset of symptoms, thus further limiting the

opportunity of obtaining viraemic patient samples for virus culture or PCR. Although the above-mentioned techniques are still considered gold standards for diagnosing CHIKV, both techniques require specialized facilities or equipment, as well as technical expertise to perform, which are too costly and impractical for widespread use.

In later phases of the illness (>1-2 weeks post-infection), ELISAs (indirect ELISAs and MAC-ELISAs) are the most commonly used tests for laboratory-based diagnoses. Other serological tests available include hemagglutination inhibition (HI), indirect ELISAs and virus-neutralization assays (Adesina and Odelola, 1991; Tiwari *et al.*, 2009; Sam *et al.*, 2011). However, most existing MAC-ELISA and HI tests are poorly established and lack credibility, in terms of specificity, due to the high possibility of false-positive results from cross-reactivity with other closely-related alphaviruses such as Ross river virus (RRV), Barmah Forest virus (BFV) and Sindbis virus (Niedrig *et al.*, 2009; Rianthavorn *et al.*, 2010; Blacksell *et al.*, 2011; Kosasih *et al.*, 2012; Reddy *et al.*, 2012). Plaque-reduction virus-neutralization tests (PRNTs) are currently recommended by the world health organisation (WHO) for serological detection of CHIKV antibodies in human. However, these assays are not only labour-intensive, but also require access to bio-containment facilities due to the handling of infectious virus, rendering them impractical for rapid and high-throughput diagnostics.

According to the World Health Organisation, the ideal test should be rapid, specific, sensitive, cost-effective, user-friendly for lesser skilled personnel, and robust in different climatic conditions. More importantly, the test should also be equipment-free (in terms of dependence on electricity) and easily accessible to those who need it (Urdea *et al.*, 2006). Herein we report the use of a monoclonal antibody (mAb) to the viral E2 protein and recombinant virus-like particles (VLPs), produced in insect cells (Metz *et al.*, 2013), in an epitope-blocking ELISA (EB-ELISA) for the sensitive and specific detection of anti-CHIKV antibodies in human sera. The use of a CHIKV-specific mAb in the assay has allowed for the development of a highly-specific diagnostic test for CHIKV that should not produce false-positive results with closely-related alphaviruses, as well as other co-circulating arboviruses such as dengue virus. Furthermore, the utilization of stably-expressed non-infectious recombinant VLPs as the coating antigen will enable the assay to be performed safely without requirements of biosafety containment. We have also illustrated the successful detection of CHIKV antigen from a human serum sample in ELISA using a combination of the CHIKV-specific mAbs. Finally, we have showed that these mAbs can be utilized in a dipstick assay for CHIKV protein detection as well. These assays offer highly-specific alternatives towards the diagnosis of CHIKV in the later stages without the issue of cross-reactivity with other closely-related alphaviruses, and can provide the basis for antigen detection in mosquito vectors as well as in the earlier phases of human CHIKV infections.

7.3. *MATERIALS AND METHODS*

7.3.1. *Cell and virus culture*

C6/36 (*Aedes albopictus* mosquito) cells were propagated in RPMI 1640 supplemented with 2% fetal bovine serum (FBS, Gibco, Life Technologies). Cultures were passaged by dissociating the cell monolayer from tissue culture flasks (Greiner Bio-One) with trypsin/PBS and were incubated at 28 °C. African green monkey kidney epithelial-derived Vero-E6 cells were cultured in DMEM (Gibco, Life Technologies) supplemented with 10% FBS. The mammalian cells were passaged by dissociating the surface monolayer with trypsin/EDTA and were cultured at 37 °C with 5% CO_2. Hybridoma cells were expanded in Hybridoma SFM (Gibco, Life Technologies) with 20% FBS at 37 °C with 5% CO_2, before being weaned off all FBS for the harvesting of mAbs in culture fluid. *Spodoptera frugiperda* (Sf21) insect cells (Invitrogen) were maintained as a monolayer culture in Grace's insect medium (Invitrogen), supplemented with 10% FBS (Hyclone). Sf9-easy titer (ET) cells (Hopkins and Esposito, 2009) were maintained as a monolayer culture in Sf-900 II (Invitrogen) serum-free medium. All cell cultures were supplemented with 50 U penicillin mL^{-1}, 50 µg streptomycin mL^{-1} and 2 mM L-Glutamine (Gibco, Life Technologies).

CHIKV viruses used in this study included the CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186) and the CHIKV La Reunion strain ($CHIKV_{IMT}$) originally isolated from a French patient returning from Reunion Island during the 2006 CHIKV outbreak (Bessaud *et al.*, 2006). CHIKV virion stocks were prepared by passaging $CHIKV_{IMT}$ twice in Vero-E6 cultures, washed with PBS, and clarified by sucrose-cushion ultra-centrifugation before storage at -80 °C. Viral plaque-forming unit assay and/or quantitative real-time PCR were utilized to evaluate the virus titres for both isolates. To obtain crude viral lysate, virus-infected C6/36 cell monolayers were incubated at 28 °C for 2-3 days before cells were rinsed in PBS and disrupted by sonication in the presence of BS9 lysis buffer (120 mM NaCl, 50 mM H_3BO_3, 1% Triton X-100 and 0.1% SDS, pH 9.0). The lysate was clarified by centrifugation at 12,000 x g for 10 min at 4 °C and stored at -20 °C (Clark *et al.*, 2007).

7.3.2. *Antigens and mAbs*

The CHIKV VLPs were expressed in insect cells using recombinant baculoviruses, and purified by affinity chromatography and sucrose gradient purification, respectively, as previously described (Metz *et al.*, 2011; Metz *et al.*, 2013). Concentrations of purified VLPs were determined by BCA Protein Assay Kit (Thermo Scientific Pierce) by the provider (Stefan Metz, personal communications) and confirmed in our laboratory with the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). CHIKV-specific anti-E2 mAbs were produced previously in Chapter 3 (Goh *et al.*, 2013),

and purified using a HiTrap Protein G HP column (GE Healthcare Life Sciences) according to the manufacturer's instructions. MAbs to be used as detecting antibodies were then biotinylated using the BiotinTag kit (Sigma), according to the manufacturer's instructions. All purified mAbs were quantified using the BCA Protein Assay Kit (Thermo Scientific Pierce).

7.3.3. *Clinical samples*

A total of 109 human serum samples were used in this study. Obtained from PathWest (PW), Laboratory Medicine, Western Australia were 10 BFV-positive, 10 RRV-positive and 30 CHIKV-positive serum samples previously collected from patients presenting with disease symptoms and infection between 6th December 1998 and 28th February 1999. These were confirmed by IgM detection via indirect immunofluorescence antibody assay, as well as HI assay (to screen for anti-IgG antibodies) (Lam *et al.*, 2001). Of the 30 CHIKV-positive PW samples, 22 were collected during the acute phase of infection, while two (PW6 and PW28) were chronic samples. The remaining 6 samples (PW9, 11, 17, 18, 24 and 26) were not able to be classified. The study also included 30 plasma samples (CHIK001-030) of patients with CHIKF who were admitted to the Communicable Diseases Centre at the Tan Tock Seng Hospital (TTSH), Singapore, from 1st August through 23rd September 2008. All patients were confirmed to have CHIKF by reverse-transcriptase PCR (Chow *et al.*, 2011). Plasma samples were collected during the late convalescent phase 4-6 weeks post-illness onset (p.i.o.) for the EB-ELISA. Clinical samples obtained from TTSH, Singapore, were approved by the National Healthcare Group's Domain-Specific Ethics Review Board (DSRB Reference No. B/08/026).

7.3.4. Epitope-blocking ELISA (EB-ELISA)

The optimal saturating concentrations of the coating antigens and detecting anti-CHIKV E2 mAb, 1.3A2, were determined using a checkerboard titration via an indirect ELISA, as described previously with modifications (Hall *et al.*, 1988; Clark *et al.*, 2007). In brief, CHIKV virions or CHIKV VLPs were titrated across U-bottom PVC 96-well plates (BD Falcon) and incubated at 4 °C overnight in coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6) or PBS, respectively, prior to detection with biotinylated 1.3A2 titrated down the plates. Optimal sub-saturating concentrations were determined by the highest OD_{405nm} value just before the absorbance reading starts to show a decrease. EB-ELISAs were performed on plates coated overnight at 4 °C with either CHIKV virions or VLPs. Coated wells were washed three times with PBS/T (0.05% (v/v) Tween-20 in PBS) and non-specific sites were blocked in TENTC blocking buffer (0.05 M Tris-HCl pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.05% (v/v) Tween 20, 0.2% (w/v) casein) for 1 h at 37 °C. Test serum samples were diluted 1/1,000 and 1/100 for the TTSH and PW samples, respectively, and added (50 µL/well) for 2 h at 37 ºC prior

to the introduction of the biotin-labelled, competing 1.3A2 at its optimal concentration, without removal of test sera, for an additional hour with gentle agitation. After rinsing six times with PBS/T, horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen) was added and the complexes were allowed to incubate for 30 min at 28 ºC. The wells were again washed before incubation with ABTS substrate solution (0.02% (w/v) 2, 2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid, 0.06% (w/v) H_2O_2 in 200 mM Na_2HPO_4 and 100 mM citric acid solution) for 1 h at 28 ºC. Optical density (OD) was measured at 405 nm using a multi-well plate reader. Results were considered valid only when the OD_{405mn} value in negative control wells were at least 0.5. Cut-off value(s) were set at the PI (percentage inhibition) for negative controls plus 3 SD values; PI = 100 - [($OD_{unknown}$ - $OD_{background}$) / ($OD_{negative\ control}$ - $OD_{background}$) x 100], where the OD_{405mn} of the negative control was the average absorbance reading of the healthy donor serum controls. PI value comparison between coating antigen used was determined by using the mean PI values of CHIKV samples, while specificity was calculated using this formula: [(No. of true positive samples + No. of true negative samples) / (Total no. of samples)] x 100%.

7.3.5. Antigen-capture ELISA

The double-antibody sandwich antigen-capture ELISA was carried out as described previously, with slight modifications – see section 3.3.9. (Goh *et al.*, 2013). Briefly, U-bottom PVC 96-well plates (BD Falcon) were coated with 1 µg/well of an anti-E2 mAb at 4 ºC overnight in coating buffer. Titrated human serum samples, at a starting dilution of 1:100, were then allowed to incubate for 1 h at room temperature, prior to an hour of blocking. A secondary biotinylated anti-E2 mAb that binds a different epitope from the capturing mAb was used as a detecting antibody. After incubating 1 h at 28 ºC, plates were washed and HRP-conjugated streptavidin (Invitrogen) was added. Following another hour of incubation, enzyme activity was visualized by the addition of ABTS substrate solution, and OD_{405mn} was measured as described above. Serum from healthy donors were used as negatives, while 20 ng/well of the sE2 protein was utilized as positive control. Results were considered valid only when the OD_{405mn} value of the positive control wells were at least 0.25 and doubled that of the negative wells. The criterion for specific recognition of antigen was defined as an OD_{405mn} value of $\geq$0.2 and at least 2-fold greater than that of the negative wells.

7.3.6. Dipstick antigen assay

The dipstick assay is a rapid immune-chromatographic, lateral flow wicking assay, modified from the method previously developed by Ryan *et al.* (2003), for the qualitative detection of CHIKV antigens from infected mosquitoes. This assay is based on a dual antibody "sandwich" principle that

uses sensitive test strips (VecTOR Test Systems Inc.). E2-specific mAbs developed previously in Chapter 3 were screened for their sensitivity and specificity (Goh *et al.*, 2013). Purified mAbs were either conjugated with colloidal gold, or immobilized as capture antibodies on the test strips (Fernandez *et al.*, 1994; Wanja *et al.*, 2014). Instead of utilizing mosquitoes grinds, $CHIKV_{ROSS}$ (GenBank ID: AF490259.3) virus culture was used in this preliminary test. The virus was prepared using the VecTOR Test grinding solution as a diluent. Test strips were then incubated with samples in their respective tubes for 30 min, after which strips were removed and examined. If present, CHIKV antigens in the mosquito tissue suspension would bind to the CHIKV-specific colloidal gold-conjugated mAb, and migrate along the test strip as a complex where it would be captured by the immobilized mAb. The development of a reddish-purple line within the "test zone" would indicate a positive result, while the appearance of an internal control line validates the performance of the assay.

7.3.7. Statistical data analysis

GraphPad Prism for Windows, version 6.00 (GraphPad Software, Inc.) was used to perform 2 or 3 standard deviation to the mean negative control OD_{405nm} to determine cut-off values for the ELISAs. Microsoft Office Excel 2013 was used to calculate the variance of PI from using whole CHIKV virions and recombinant CHIKV VLPs as coating antigens in the EB-ELISA.

7.4. RESULTS

7.4.1. Detection of CHIKV by E2-specific mAbs

Preliminary testing was carried out to determine the affinities of five biotin-labelled CHIKV E2-specific mAbs, designated 1.3A2, 4.6F5, 4.10C12, 5.2B2 and 5.2H8, against the two coating antigens used for this study - CHIKV VLPs and live, infectious virions - in an indirect ELISA. Biotinylated mAb 1.3A2, at its optimal sub-saturating concentration of 1.25 µg/mL, showed the strongest interaction with both the CHIKV VLPs and infectious virions, with OD_{405nm} readings of >1.0 (results not shown). Furthermore, biotinylated 1.3A2, 4.6F5 and 4.10C12 were inhibited more efficiency than mAbs 5.2B2 and 5.2H8 in EB-ELISA in the presence of serially-diluted anti-CHIKV polyclonal mouse serum (results not shown). We have shown in Chapter 3 that mAbs 1.3A2, 4.6F5, and 4.10C12 exhibit complete two-way inhibition of each other in competitive binding studies indicating that they bind the same or adjacent epitopes within the same region of the E2 protein (Goh *et al.*, 2013). Considering these results, and our observation that the 1.3A2 hybridoma line was a superior producer of mAb, we selected this antibody for further analysis.

7.4.2. Development of an EB-ELISA

Results from checkerboard titrations of CHIKV antigens and detecting mAb revealed that the optimal concentrations of recombinant VLP or purified CHIKV virion antigen were 200 ng or 10^6 infectious units per well, respectively (results not shown). Initially we compared the effectiveness of using CHIKV VLPs to that of using CHIKV virions as antigens in the EB-ELISA using serial dilutions of three reference samples; PW01 (CHIKV-immune); PW31 (RRV-immune) and PW41 (BFV-immune). Using infectious virions at their optimal coating concentration, PI exhibited by the CHIKV-immune sample declined significantly for each doubling dilution, with 1:400 diluted sera producing ~50% inhibition (Figure 7.1). By comparison, using CHIKV VLP antigen in the EB-ELISA revealed >50% inhibition with the same CHIKV-positive sample diluted to 1:3,200. These results indicated that the VLP antigen was at least as sensitive as the native virion in this format.

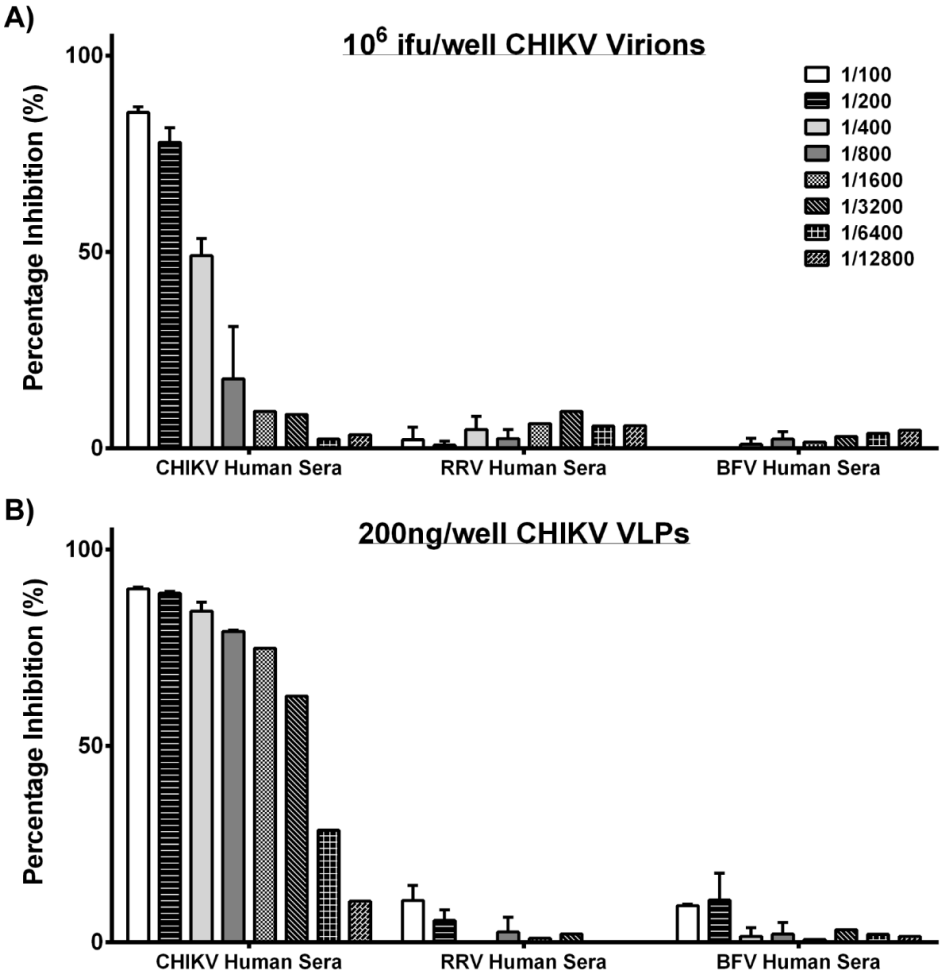


Figure 7.1. Epitope-blocking ELISA analyses of CHIKV, RRV and BFV-infected patient sera using (A) CHIKV virions, or (B) CHIKV VLPs. C6/36 cell lysate and baculovirus-expressed GFPs were used as negative control antigens, respectively (results not shown).

To determine a suitable cut-off value for PI in the EB-ELISA, a panel of four sera from healthy donors lacking antibodies to CHIKV - based on IFA for anti-CHIKV IgM - were tested for non-specific inhibition of mAb 1.3A2 binding to VLPs or virions. The mean non-specific PI of negative samples using virion antigen was 7.9% with a 3 x SD of 7.7%, while the PI by these samples on VLP antigen was 5.7% with a 3 x SD of 12.3%. Using a criteria of "mean PI of negative sera plus 3 x SD" this corresponds to cut-off values of 15.6% and 18%, respectively. A fixed cut-off point of 20% was set, with serum samples exhibiting PI of between 20% and 30% considered "positive with requirement of confirmation by RT-PCR, virus culture and/or neutralization", which is a common practice for serological diagnostic assays.

7.4.3. Evaluation of EB-ELISA using clinical serum samples

The EB-ELISA demonstrated a sensitivity of 100% with all 60 CHIKV-immune serum, previously confirmed by IgM immunofluorescence and HI assays, also testing positive in the EB ELISA (Table 7.1). The frequency distribution of percentage inhibition based on the use of CHIKV virions as coating antigen showed that more than two-thirds of the samples exhibited >60% inhibition (Figure 7.2). The EB-ELISA also exhibited 98.5% specificity when CHIKV virions were used as antigen to assess inhibition by 10 RRV- and 10 BFV-immune sera, with a single RRV-positive sample exhibiting a PI of 22.7%. When VLPs were being used as the coated antigen, 100% specificity was obtained with the same samples (Figure 7.3). To further compare the efficiency of using CHIKV virion antigen with that of recombinant VLPs, mean PIs were recorded for 30 CHIKV-immune plasma samples tested in the EB-ELISA. The samples gave an average PI of 61.7% when virion antigen was used and 58.2% when VLPs were employed (Figure 7.3). These values indicated the two antigens provided similar efficacy in the EB-ELISA format.

Table 7.1. Comparison of the CHIKV-specific EB-ELISA against standard diagnostic techniques used for detection of antibodies to CHIKV in human sera.

Human Serum Sample ǂ	Virus Type	PI (Virions)	PI (VLPs)	HI Titre	Human Serum Sample ǂ	Virus Type	PI (Virions)	PI (VLPs)	HI Titre
CHIK001	CHIKV	74.4	N.D.	N.D.	PW11	CHIKV	52.0	54.5	320
CHIK002	CHIKV	75.6	N.D.	N.D.	PW12	CHIKV	44.9	59.6	160
CHIK003	CHIKV	67.6	N.D.	N.D.	PW13	CHIKV	43.2	64.2	160
CHIK004	CHIKV	56.4	N.D.	N.D.	PW14	CHIKV	36.8	59.2	<10
CHIK005	CHIKV	78.3	N.D.	N.D.	PW15	CHIKV	47.2	74.2	160
CHIK006	CHIKV	85.4	N.D.	N.D.	PW16	CHIKV	56.4	61.6	320
CHIK007	CHIKV	77.3	N.D.	N.D.	PW17	CHIKV	65.9	76.5	160
CHIK008	CHIKV	82.0	N.D.	N.D.	PW18	CHIKV	39.4	44.7	40
CHIK009	CHIKV	84.2	N.D.	N.D.	PW19	CHIKV	71.0	65.6	320
CHIK010	CHIKV	78.9	N.D.	N.D.	PW20	CHIKV	60.9	56.1	640
CHIK011	CHIKV	79.4	N.D.	N.D.	PW21	CHIKV	76.1	60.9	160
CHIK012	CHIKV	88.8	N.D.	N.D.	PW22	CHIKV	43.9	73.1	640
CHIK013	CHIKV	70.7	N.D.	N.D.	PW23	CHIKV	36.1	47.8	320
CHIK014	CHIKV	79.8	N.D.	N.D.	PW24	CHIKV	76.8	45.8	>640
CHIK015	CHIKV	71.3	N.D.	N.D.	PW25	CHIKV	41.8	64.3	80
CHIK016	CHIKV	61.5	N.D.	N.D.	PW26	CHIKV	78.6	54.5	320
CHIK017	CHIKV	81.1	N.D.	N.D.	PW27	CHIKV	72.7	54.8	160
CHIK018	CHIKV	85.8	N.D.	N.D.	PW28	CHIKV	83.3	80.7	320
CHIK019	CHIKV	61.5	N.D.	N.D.	PW29	CHIKV	73.9	57.3	80
CHIK020	CHIKV	84.1	N.D.	N.D.	PW30	CHIKV	77.1	47.8	40
CHIK021	CHIKV	76.5	N.D.	N.D.	PW31	RRV	0.0	6.2	40
CHIK022	CHIKV	59.4	N.D.	N.D.	PW32	RRV	0.0	11.2	20
CHIK023	CHIKV	83.4	N.D.	N.D.	PW33	RRV	0.0	16.0	160
CHIK024	CHIKV	90.4	N.D.	N.D.	PW34	RRV	0.0	7.8	160
CHIK025	CHIKV	84.8	N.D.	N.D.	PW35	RRV	0.0	10.3	<10
CHIK026	CHIKV	86.9	N.D.	N.D.	PW36	RRV	0.0	3.5	160
CHIK027	CHIKV	77.3	N.D.	N.D.	PW37	RRV	16.2	6.3	40
CHIK028	CHIKV	83.3	N.D.	N.D.	PW38	RRV	12.8	7.6	40
CHIK029	CHIKV	81.5	N.D.	N.D.	PW39	RRV	22.7	0.8	160
CHIK030	CHIKV	80.9	N.D.	N.D.	PW40	RRV	3.6	0.0	160
PW01	CHIKV	78.5	46.5	320	PW41	BFV	0.0	0.0	40
PW02	CHIKV	60.7	46.0	160	PW42	BFV	0.0	0.0	160
PW03	CHIKV	64.0	45.9	80	PW43	BFV	0.0	3.6	>640
PW04	CHIKV	87.5	50.1	20	PW44	BFV	17.3	7.5	160
PW05	CHIKV	82.2	59.6	320	PW45	BFV	0.0	0.0	>640
PW06	CHIKV	42.4	60.1	640	PW46	BFV	0.0	0.0	40
PW07	CHIKV	75.8	58.3	640	PW47	BFV	0.0	6.7	80
PW08	CHIKV	63.3	57.7	640	PW48	BFV	10.2	1.4	40
PW09	CHIKV	69.8	65.4	80	PW49	BFV	9.2	0.0	160
PW10	CHIKV	47.6	53.9	320	PW50	BFV	0.0	0.0	40

ǂ Samples CHIK001-030 were tested CHIKV-positive by RT-PCR (Chow et al., 2011), while PW samples were confirmed to be CHIKV-positive by HI and IFA via IgM-detection.

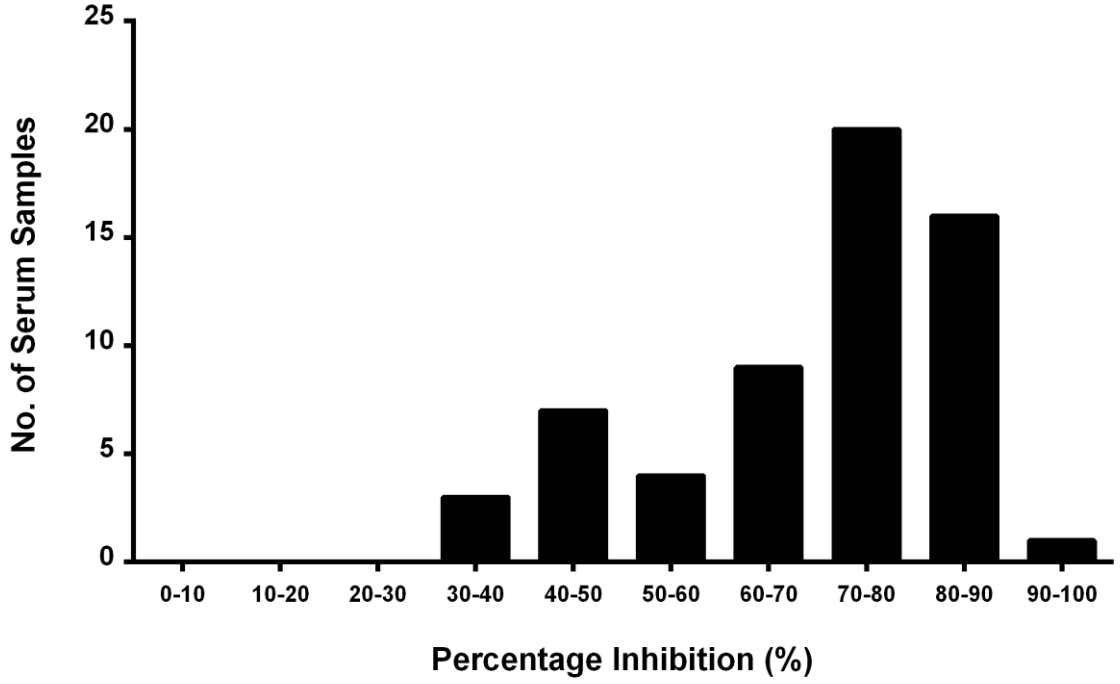


Figure 7.2. Overall frequency distribution of PI values based on the EB-ELISA results from the 60 CHIKV-positive human samples tested with CHIKV virions used as coating antigen. Serum samples were considered positive for CHIKV antibodies when PI > 30%, and negative when PI < 20%. Serum samples exhibiting PI of between 20% and 30% were considered "positive with requirement of confirmation by virus culture and/or neutralization".

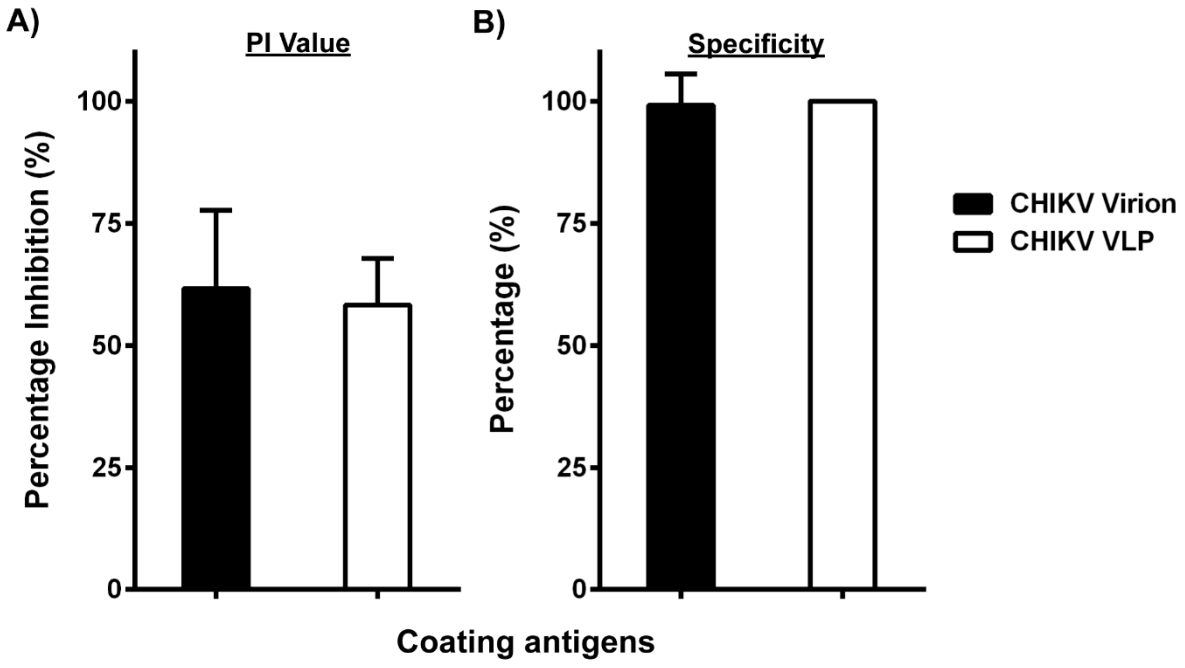


Figure 7.3. Comparison of PI values and specificity of using CHIKV virions vs. VLPs in EB-ELISA. Thirty CHIKV-positive patient samples were utilized in the EB-ELISA for the comparison of A) sensitivity, in terms of PI, and B) specificity of using CHIKV virions vs. CHIKV VLPs as coating antigen.

7.4.4. Use of anti-E2 mAbs for detection of CHIKV antigens

Our previous results (see Chapter 3) involving the generation of anti-CHIKV mAbs has shown the potential of utilizing a combination of two mAbs that map to different sites of the E2 protein in a double-antibody sandwich antigen-capture ELISA to detect for the presence of CHIKV in human blood samples (Goh *et al.*, 2013). Monoclonal antibody 5.2B2 was coated onto 96-well plates for the capture of CHIKV E2 proteins, while a non-competing, biotinylated mAb 5.2H8 was used as the detecting antibody. Out of 29 RT-PCR positive patient samples tested, only one tested positive in the ELISA (Figure 7.4). The remaining 28 samples exhibited absorbance readings that were greater than that elicited by the negative controls at their respective dilutions. However, their OD_{405nm} values were not at least twice that of the healthy donor samples and were thus deemed negative.

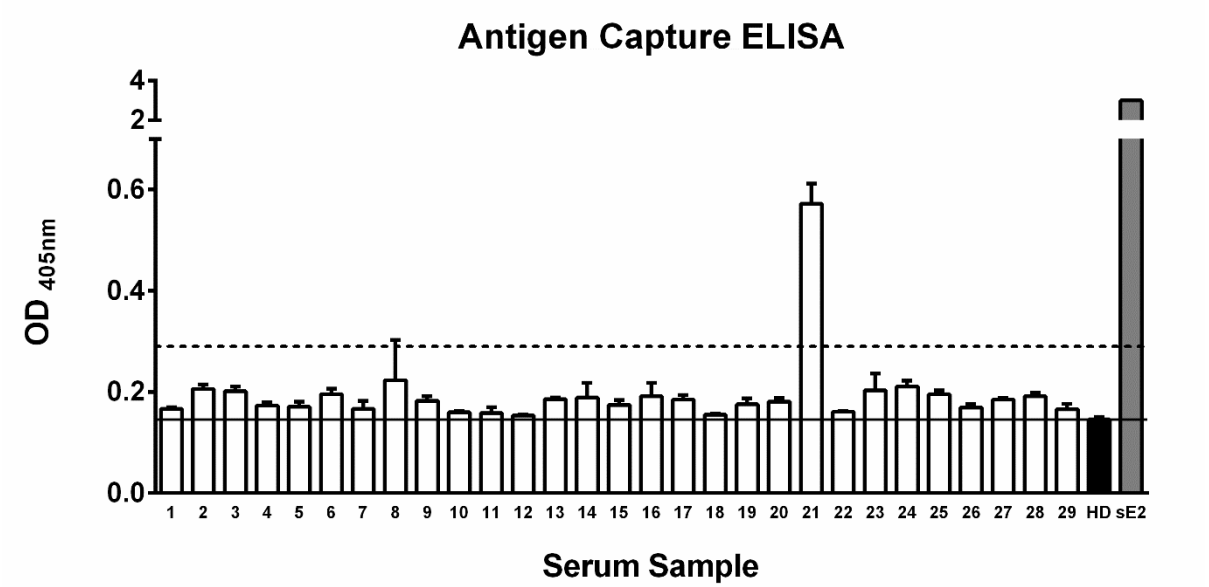


Figure 7.4. Detection of CHIKV-specific protein in a human serum sample using an antigen-capture ELISA. Unlabelled mAb 5.2B2 was coated onto 96-well plates at 1 µg per well prior to incubation with diluted clinical samples. The detecting antibody, biotinylated mAb 5.2H8, was used at 400 ng per well. The mean OD_{405nm} values of three replicates were plotted with bars showing SD. Protein sE2 (n = 20) was used as a positive control, while healthy donor serum sample (HD; n = 20) was utilized as a negative control for the assay. Solid line represents the mean negative OD_{405nm} value, while the dotted line indicates the cut-off point, derived from 2 x the mean negative OD_{405nm} reading.

The mAbs were then incorporated into a dipstick assay, by our collaborators at VecTOR Test Systems Inc., to determine if the dipstick antigen-capture format could be used for the detection of viral proteins in diluted samples. Paired combinations (for capture and detection) of the five anti-E2 mAbs – labelled B1, B3, A2, A3, C1 and C2 – were kept confidential due to commercialization reasons. Nonetheless, results from testing indicated that some combinations performed better than the others

(Figure 7.5). From highest to lowest signal intensity: B3, B1, A2, C1, C2 and A3. Combination B3 was then used to test a host of other closely-related alphaviruses (including CHIKV), as well as several unrelated flaviruses. Further testing also revealed that combination B3 managed to specifically detect CHIKV proteins, but not from any of the other 14 viruses tested (Figure 7.6).

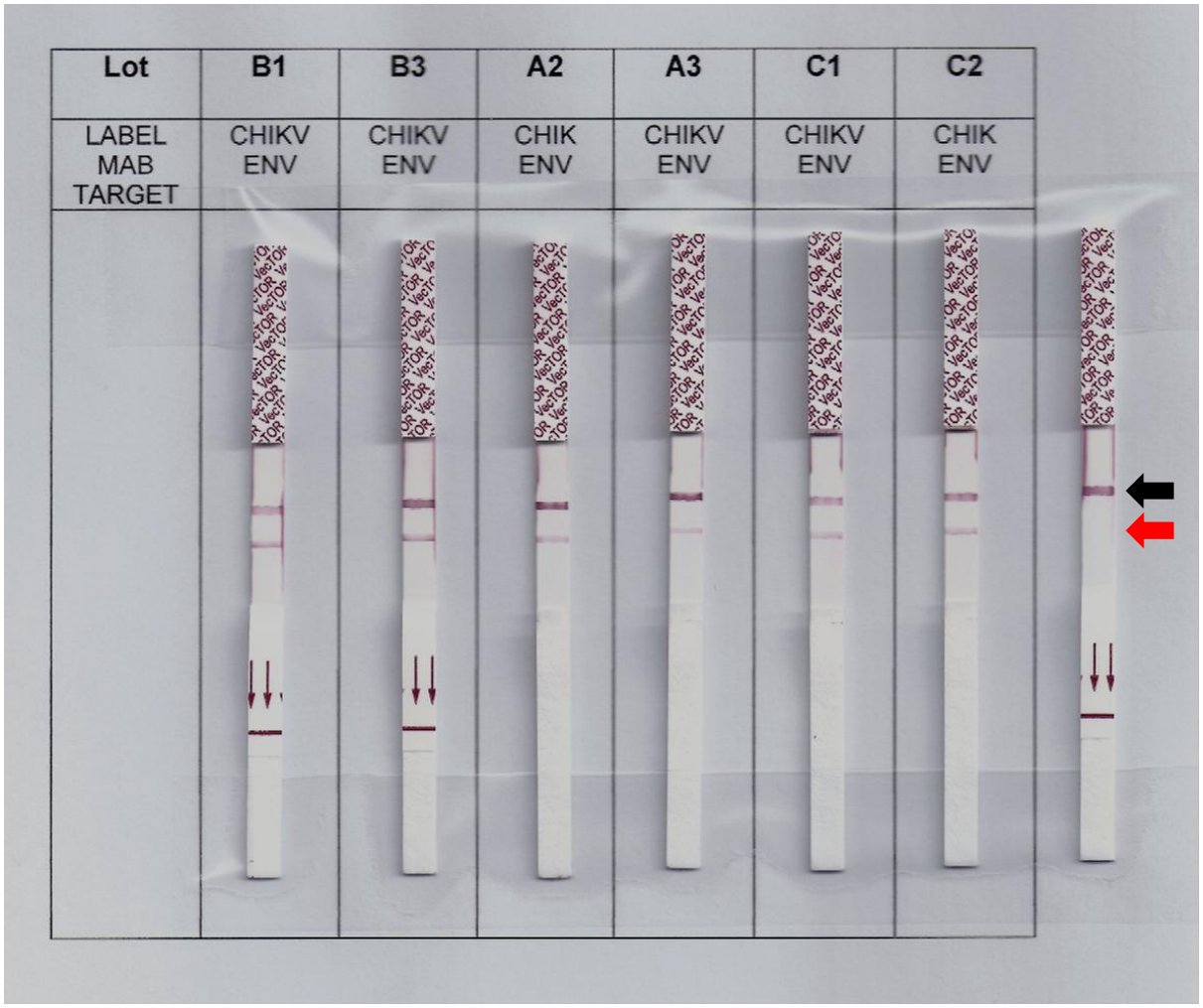
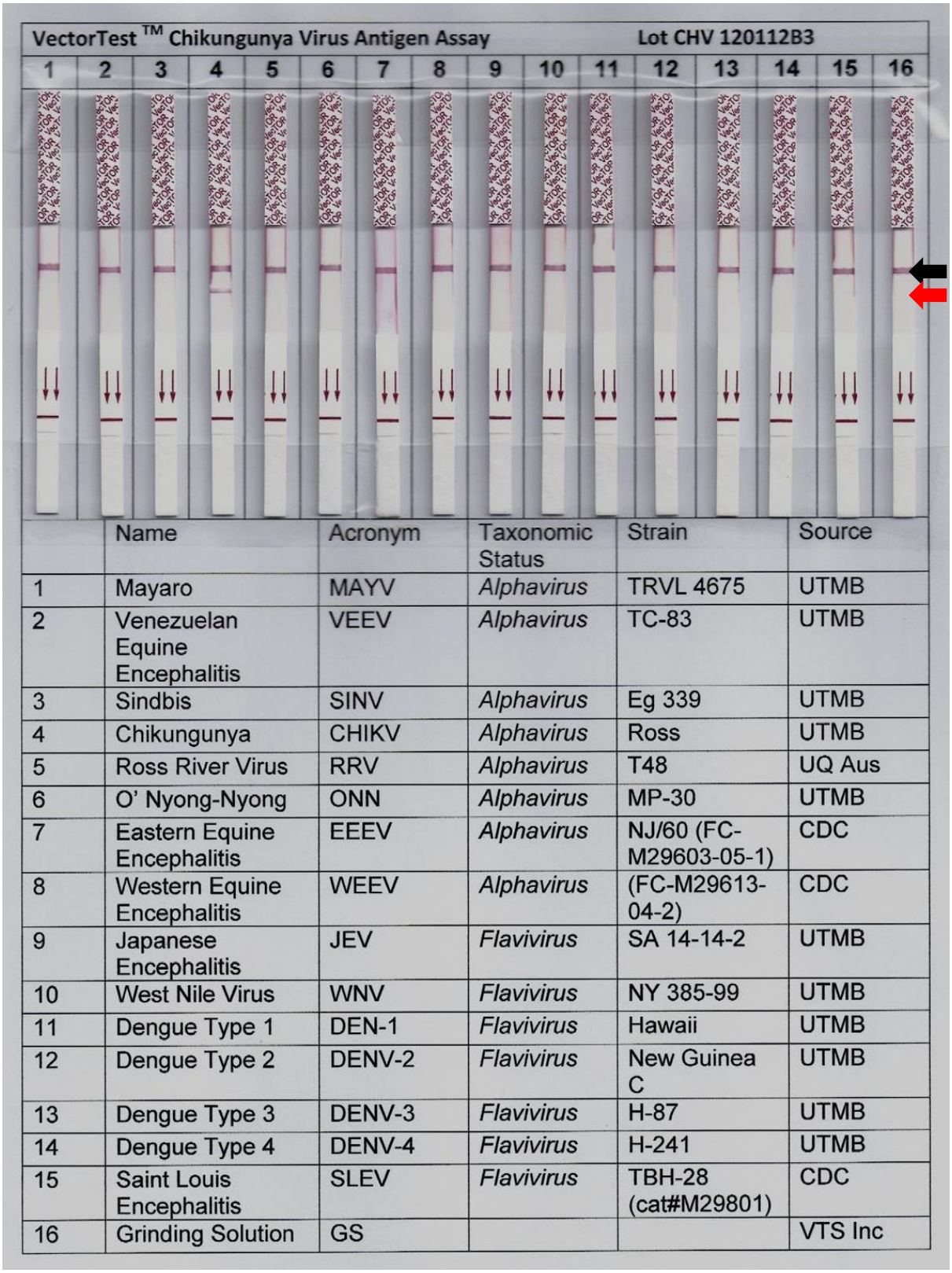


Figure 7.5. Detection of CHIKV antigens via dipstick assay. Six combinations of the anti-E2 mAbs as capture and detecting antibody were used for the detection of CHIKV proteins in diluted samples. Top control line (black arrow) acts as a positive control to indicate that the test strip is functioning properly, while bottom line (red arrow) is the test line.



	Name	Acronym	Taxonomic Status	Strain	Source
1	Mayaro	MAYV	*Alphavirus*	TRVL 4675	UTMB
2	Venezuelan Equine Encephalitis	VEEV	*Alphavirus*	TC-83	UTMB
3	Sindbis	SINV	*Alphavirus*	Eg 339	UTMB
4	Chikungunya	CHIKV	*Alphavirus*	Ross	UTMB
5	Ross River Virus	RRV	*Alphavirus*	T48	UQ Aus
6	O' Nyong-Nyong	ONN	*Alphavirus*	MP-30	UTMB
7	Eastern Equine Encephalitis	EEEV	*Alphavirus*	NJ/60 (FC-M29603-05-1)	CDC
8	Western Equine Encephalitis	WEEV	*Alphavirus*	(FC-M29613-04-2)	CDC
9	Japanese Encephalitis	JEV	*Flavivirus*	SA 14-14-2	UTMB
10	West Nile Virus	WNV	*Flavivirus*	NY 385-99	UTMB
11	Dengue Type 1	DEN-1	*Flavivirus*	Hawaii	UTMB
12	Dengue Type 2	DENV-2	*Flavivirus*	New Guinea C	UTMB
13	Dengue Type 3	DENV-3	*Flavivirus*	H-87	UTMB
14	Dengue Type 4	DENV-4	*Flavivirus*	H-241	UTMB
15	Saint Louis Encephalitis	SLEV	*Flavivirus*	TBH-28 (cat#M29801)	CDC
16	Grinding Solution	GS			VTS Inc

Figure 7.6. Specificity of combination B3 in CHIKV dipstick assay. Fifteen different viruses were tested for their reactivity against the anti-E2 mAbs (B3 combo), with VecTOR Test Systems Inc. grinding solution as the negative control. Top control line (black arrow) acts as a positive control to indicate that the test strip is functioning properly, while bottom line (red arrow) is the test line.

7.5. DISCUSSION

Epitope-blocking ELISAs have been successfully used for the sensitive and specific detection of serum antibodies to a variety of viral pathogens, including RRV, West Nile virus and influenza (Hall *et al.*, 1995; Blitvich *et al.*, 2003; Oliveira *et al.*, 2006; Lorono-Pino *et al.*, 2009; Prabakaran *et al.*, 2009; Sotelo *et al.*, 2011). This study describes the use of a mAb that recognizes an epitope on the CHIKV E2 protein in an EB-ELISA to detect CHIKV-specific antibodies in clinical specimens.
Sixty serum samples from individuals with known CHIKV exposure were utilized to evaluate the performance of the EB-ELISA. Using biotinylated 1.3A2 as the detecting antibody, the blocking ELISA detected CHIKV-specific antibodies in all convalescent human sera (100% sensitivity), with no false-positive results apart from a single RRV-positive sample, PW39, that exhibited low levels of inhibition (22.7%) of 1.3A2 binding, deeming it "positive with requirement of confirmation by virus culture and/or neutralization". The RRV and BFV human serum samples were not previously tested for presence of antibodies to CHIKV. Therefore, although unlikely, the possibility that sample PW39 was obtained from a patient who had low levels of CHIKV antibodies from a prior asymptomatic exposure exists. Nonetheless, this still reflects a 98.5% specificity for the EB-ELISA using the 20% PI cut off.

The anti-CHIKV mAb utilized in this study (1.3A2) was previously shown to have no cross-reactivity with other alphaviruses tested – see section 3.4.1. (Goh *et al.*, 2013). This high level of specificity is consistent with the lack of inhibition of the mAb in the EB-ELISA in the presence of serum antibodies from patients with confirmed RRV or BFV infections. Thus the CHIKV-specific EB-ELISA developed in this study provides significant improvement in terms of specificity, allowing it to be used in regions of the world, such as Australia, Europe, Africa and Central/South America, where other alphaviral diseases of humans occur (Suhrbier *et al.*, 2012; Knope *et al.*, 2013).

MAb 1.3A2 was also previously shown to recognise CHIKV isolates that represent the two major global lineages of the virus (Asian and East/Central/South African) – see section 3.4.1. The highly-conserved nature of the E2 epitope recognized by this mAb is also predicted to provide similar detection sensitivity of antibodies to a range of CHIKV strains in the EB-ELISA. This is particularly relevant to patient samples from the Caribbean, where a CHIKV strain of the Asian genotype is suggested to be responsible for the ongoing outbreak (Lanciotti and Valadere, 2014).

The recombinant CHIKV VLP antigen assessed in this study was previously generated in a well-established baculovirus expression system as a safe and non-replicating vaccine candidate, and its production can be similarly scaled up for diagnostic use (Metz *et al.*, 2013). More importantly, CHIKV VLPs exhibited a sensitivity comparable to that of infectious virions when used as the coating

antigen in the EB-ELISA. When 30 CHIKV-positive serum samples were used to compare the efficacy of the two antigens less than 3% variance was observed, with mean PIs of 61.7% and 58.2% for virions and VLPs respectively. This comparison demonstrated that apart from being safe, stable and easily mass-produced, the recombinantly-expressed CHIKV VLPs are suitable for replacing native CHIKV virions as antigens in this ELISA format.

An antigen-capture ELISA was also developed for the detection of CHIKV proteins in human sera. By utilizing a pair of the anti-CHIKV E2 mAbs, a sandwich ELISA was designed to be used in conjunction with an antibody-detecting assay to significantly increase the window of diagnosis a CHIKV-infected patient. It is well understood that during the early stages of CHIK fever, where most patients seek medical advice, antibody responses might not be at sufficient levels required for detection due to the delayed activation of the humoral response (Suhrbier *et al.*, 2012). This is when blood is taken to obtain a diagnosis, generally via virus culture and/or molecular techniques. The availability of an antigen-capture ELISA can be helpful in assisting clinicians obtain a preliminary diagnosis, prior to further testing. Safe, rapid and easy-to-perform, the antigen-capture assay could be used as a frontline test to screen for potential CHIKV-positive cases that will allow for more efficient quarantine and control measures, if required. The antigen-capture ELISA was previously tested for its ability to detect CHIKV antigens in the form of the sE2 protein (see section 3.4.5.), and was found to be capable of detecting antigen in the picogram range (Goh *et al.*, 2013). Herein, clinical serum samples, collected at an average of 4 days p.i.o., were used to evaluate the assay. Results showed that only one out of 29 samples tested positive for the presence of CHIKV proteins. However, all samples exhibited reactivity above the healthy donor negative control. Due to the limited quantity of human samples available, samples were diluted 1:100, which might have been a crucial factor in the result, considering most antigen-capture assays require serum samples to be used neat or at a maximum dilution of 1:10 (Shukla *et al.*, 2009; Yathi *et al.*, 2013). Nonetheless, this result has illustrated the potential of these CHIKV E2-specific mAbs to detect minute quantities of viral protein, and as shown in the dipstick assay, may have potential as a vector surveillance tool pending further testing on mosquito samples.

7.6. CONCLUSIONS

The EB-ELISA for CHIKV diagnosis described here represents a rapid, simple, highly-sensitive and specific assay that is also cost-effective and safe. These attributes potentially meet the criteria set by the WHO for an ideal diagnostic test for CHIKV and may be utilized as a rapid front-line screening assay for the serodiagnosis of CHIKF. Furthermore, the platform's robustness will enable it to be used in harsher environments as a point-of-care test. Lastly, the dipstick assay has suggested another

potential use for these mAbs as reagents for the specific detection of CHIKV protein, especially in mosquito vectors.

7.7. *ACKNOWLEDGEMENTS*

CHIKV VLPs were produced within the framework of Dutch Top Institute Pharma, project "Development of recombinant live and subunit vaccines against Chikungunya virus infections" (project nr. T4-301). Lucas Yuan Hao Goh was supported by the UQIRTA from the UQ Graduate School, The University of Queensland, and the ANZ Trustees scholarship for medical research, Queensland.

CHAPTER 8: GENERAL DISCUSSIONS AND CONCLUSION

The primary objective of this project was to develop, characterise and evaluate a set of reagents, in the form of mAbs and recombinant antigens, that would be useful for CHIKV research, diagnostics and potentially as therapeutics to prevent disease. This thesis has described the generation of hybridomas secreting mAbs specific for the capsid and E2 protein of CHIKV. These mAbs were evaluated for their ability to detect CHIKV in various assays such as ELISA, Western blot, IFA and immunohistochemistry. The work presented in this study has shown that the anti-E2 mAbs have potential prophylactic properties while the CP-specific mAbs were capable of recognising multiple isolates of CHIKV as well as several other closely-related alphavirus such as RRV, BFV and SINV. In addition, these CHIKV E2-specific mAbs demonstrated their effectiveness, in the format of an EB-ELISA, for the detection of anti-CHIKV antibodies in patients. Finally, the anti-E2 mAbs also showed their potential use in the capture of CHIKV antigen in both human and mosquito samples.

Data obtained whilst attempting to determine the binding sites of the anti-CHIKV CP mAbs suggested that the C-terminus of CP is essential for antibody binding. Further analysis of native and recombinant CP also confirmed the presence of minor, N-terminally truncated versions of the protein, as seen in Figures 5.6 and 5.7. Lastly, using a novel technique to avoid the amplification of dominant non-functional mRNA transcripts, a chimeric mouse/human mAb was engineered and transiently expressed to assess its potential use as an alternate positive control reagent in ELISA kits for the diagnosis of CHIKV.

The re-emergence of CHIKV and its continued spread into previously unaffected regions has demonstrated that it is a serious public health problem on a global scale. Furthermore, the disease it causes can have a significant social and economic impact on affected communities. The high epidemic potential of CHIKV further accentuates the exigency for accurate diagnostics and effective anti-viral therapeutics (Akahata *et al.*, 2010; Wang *et al.*, 2011; Brandler *et al.*, 2013; Metz *et al.*, 2013; Powers, 2014).

8.1. GENERATION OF CHIKV-SPECIFIC MABS

The generation of anti-CHIKV mAbs was carried out to meet the current shortage of research and diagnostic reagents for CHIKV. Following intensive screening of the antibody-producing hybridomas, mAbs harvested from selected and cloned parental cell lines were further characterised using multiple assays and were shown to bind either to the E2 or CP of CHIKV (Goh *et al.*, 2013; Goh *et al.*, 2014). The utility of these generated mAbs was highlighted when it was determined that

the anti-E2 mAbs were specific for CHIKV, but reacted equally well with isolates form different genotypes. In contrast, the anti-CP mAbs cross-reacted with related alphaviruses, including RRV, SINV and SFV.

8.2. E2-SPECIFIC MABS

A representative anti-E2 mAb, 1.3A2, was shown to prevent CHIK disease in mice administered with the mAb prior to challenge with the virus. Although its use in treating human disease may be impractical considering the large quantities that will probably be required for this antibody, this model may serve as a platform for the development of more potent mAb therapeutics/prophylactics against CHIKV and other medically important alphaviruses. For example, virus neutralising antibodies have been suggested for preventing vertical mother-to-child transmissions. MAbs are also currently being used in the prophylaxis of several infections, such as hepatitis A virus, hepatitis B virus, and respiratory synctial virus infections in the form of passive immunisation, prior to expected exposure, such as a forecasted epidemic or the entering of a region known to be endemic to certain infectious diseases (Sawyer, 2000; Both *et al.*, 2013). Progress in the development of inexpensive production platforms will furthermore allow antiviral mAbs to be more widely available and affordable. Due to the non-conformational nature of the anti-E2 mAbs, we were unable to map their binding sites within the protein. However, it is probable that these mAbs recognises either the receptor-binding site(s) of E2 or an epitope capable of interfering with virus attachment onto the host cell membrane. Competitive binding of the E2-specific mAbs revealed that they recognised at least three spatially separated epitopes on the protein. This information was then used to design a capture assay capable of detecting CHIKV proteins in samples. Coupled with their high specificity towards the virus, the E2-specific mAbs were demonstrated to be extremely useful for the detection of CHIKV antigens in mosquito vectors. The rapid detection of CHIKV in mosquitoes via the use of a dipstick assay can provide a rapid and convenient surveillance tool for the prompt assessment of disease threat and timely implementation of vector control measures. These strategies are particularly useful during military deployments in high-risk areas (Nasci *et al.*, 2003; Sattabongkot *et al.*, 2004; Coleman *et al.*, 2009; Wanja *et al.*, 2014). However, when clinical samples positive for CHIKV RNA by RT-PCR were tested in the antigen-capture assay, the efficiency of CHIKV antigen detection was relatively poor, with only one out of 29 early-phase human samples positive. Although all remaining samples exhibited reactivity above the healthy donor negative control, their OD_{405nm} values did not reach the cut-off threshold (at least double that of the healthy donor samples) which was statistically determined to reduce the chance of false positives. The limited volume of human samples available, which required dilution to 1:100 for use in the assay, may have reduced our ability to detect viral antigen in

these samples. Indeed, most antigen-capture assays require serum samples to be used neat or at a maximum dilution of 1:10 (Shukla *et al.*, 2009; Yathi *et al.*, 2013). While the data has illustrated the potential of these CHIKV E2-specific mAbs to detect minute quantities of viral protein, further optimization of the antigen capture assay with lower dilutions of samples is required to fully assess the sensitivity and potential of the assay for clinical use.

In this thesis we also demonstrated that one anti-E2 mAb, 1.3A2, exhibited exceptional sensitivity for the detection of CHIKV-specific antibodies in human serum samples in EB-ELISA. Both live CHIKV virions and recombinant CHIKV VLPs were assessed as coating antigens in the ELISA, and while no significant difference in the sensitivity or specificity were observed between the two antigens, we were unable to make a direct comparison as a result of the different dilutions of clinical samples used for each experiment, due to a lack of serum volume for the TTSH (Singapore) samples. Nevertheless, the ability of the EB-ELISA to retain its sensitivity and specificity even when the TTSH samples were diluted 1:1,000, instead of 1:100, further illustrates the high sensitivity of the detection assay using both antigens. More importantly, the successful use of the CHIKV VLPs as a non-infectious antigen, that mimics the native structure of the virus and can be stably and continuously expressed on a large scale, has provided a safe and efficient alternative to using live virus in diagnostic assays. In conclusion, this use of an EB-ELISA for diagnosing CHIKF represents a safe, robust, simple-to-use and cost-effective alternative to currently available commercial kits (e.g. indirect and MAC-ELISAs).

8.3. CP-SPECIFIC MABS

We have shown with multiple assays such as ELISA, IFA, Western blot and IHC, that the anti-CP mAbs are capable of sensitive recognition of CHIKV antigen. The successful application of a representative mAb, 5.5G9, to detect CHIKV antigen in murine tissue samples in IHC further reinforces the potential use of these CP-specific mAbs for clinical diagnostics and research purposes, including the study of viral pathogenesis. Further evaluation of these mAbs against other medically significant alphaviruses will only increase their value as diagnostic tools in other regions of the world.

Aside from their use in the identification of CHIKV, we believe that further characterising these CP-reactive mAbs, in terms of their binding sites on the protein, will enable us to gain a better idea of how we can utilise these antibodies to obtain more information on this essential component of the nucleocapsid. Initial results indicated that anti-CP mAbs recognised linear epitopes by reacting with reduced and carboxymethylated antigens in Western blot. The use of recombinant C- or N-terminally truncated versions of CP further suggested that the group 1 mAbs recognised a region spanning

approximately 105 aa (CP 105-210), while the group 2 mAbs bound within the first 35 aa of CP. These findings were supported by mass spec analysis of truncated native proteins in CHIKV-infected lysates that were bound by group 1 mAbs which further defined their binding region between residues 157 and 210.

To further narrow down the binding sites of these mAbs we designed synthetic peptides encompassing the entire CHIKV CP and assessed their recognition by the anti-CP mAbs in several assays, including ELISA and MIA. Unfortunately, none of the synthetic peptides were bound by CP-specific mAbs or polyclonal anti-CHIKV mouse sera, despite all control peptides, that were synthesised in the same batch, being recognised by their respective mAbs in both assays. One conclusion from this result was that post-translational phosphorylation, which does not occur with synthetic peptides, was required for binding, consistent with the existence of potential phosphorylation sites in both of the predicted binding regions for these mAbs. However, the puzzling observation that none of the C-terminally truncated recombinant CP proteins were recognised by anti-CP mAbs (even the group 2 mAbs predicted to bind to the N terminal region) or anti-CHIKV polyclonal serum, was inconsistent with this hypothesis. A second conclusion was that the C-terminus of CP is required for the protein to fold into an antigenically-authentic structure and allow antibody binding to occur. Based on the position of the smallest C-terminal truncation, this would suggest that the last 35 residues are required for recognition of the capsid by the mAbs or polyclonal antibodies used in this study. Without a doubt, this deduction would be compatible with the predicted binding sites of the 2 groups of mAbs and their failure to recognise the synthetic peptides. Future studies using rCap with internal deletions and various lengths of intact C-terminal sequences would confirm this hypothesis and identify the minimum C-terminal sequence required for mAb binding and elucidate the associated mechanism.

In the process of mapping the epitopes of CP-specific mAbs, we also made an observation on the presence of a smaller, truncated version of CP in Western blots. Minor truncated products of the CP have previously been identified as unknown proteins or simply as “protein X” in CHIKV and some other alphaviruses (Bell *et al.*, 1983; Choi *et al.*, 1996; Cho *et al.*, 2008; Brandler *et al.*, 2013). While the presence of sCP has not been established in CHIKV, the discovery of a minor CP was recorded in WEEV and SINV (Ishida and Simizu, 1981; Choi *et al.*, 1996), with Choi and colleagues suggesting a truncation of 113 N-terminal residues within the SINV CP. We have since confirmed by reactivity, with the help of the anti-CP mAbs, that the ~24 kDa protein is a part of the CP. Choi *et al.*’s (1996) prediction that the sCP was reduced by 100-110 aa also accurately reflects its size of ~24 kDa on Western blot. The binding of the group 1 mAbs, but not the group 2 mAb, towards the sCP further supports this theory, while the missing residues also led us to propose that this product is

potentially a result of an alternate translation initiation codon, predicted to occur at position 105 in CHIKV CP. This is further supported by mass spectrometry analysis of CP molecules isolated from infected cell lysates by immune-precipitation with group 1 mAbs. While full-length and smaller versions of CP retained the same peptides at the C-terminal end of the protein, the smaller species of CP lacked peptides at the N-terminus. Indeed, the first six residues representing an N-terminal peptide of a major truncated species, MCMKIE (corresponding to CP 105-110), is predicted to be translated from the putative alternative translation initiation site.

The fact that this minor product of the CP has been consistently detected in numerous alphaviruses, in both recombinant and native forms of the protein, implies that it may have functional significance during infection. Although we were unable to fully characterise this little-known sCP, the availability of these mAbs has allowed for further proteomic experimentations that were previously not possible. The anti-capsid mAbs have also been used to demonstrate nuclear staining within cells infected with CHIKV or transfected with rCap, supporting recent literature that the CHIKV CP contains nuclear import and export signals (Thomas *et al.*, 2013). Reasons behind the transport of CP - and potentially sCP - to and from the host cell nucleus is unknown, and thus accentuates the requirement for further research into the role and function of the CHIKV CP.

8.4. CHIMERIC MAB 5.2H7

The versatility of the CP-specific mAbs led us to creation of a genetically re-engineered humanised version of the 5.2H7 mAb that could be utilised as a positive control reagent, providing diagnostic kits with a specific, consistent, cost-effective and ethical alternative to using human reference sera. Interestingly, through a minor but significant malfunction of a commercial antibody isotyping kit, we have managed to unveil a novel mass spectrometry-guided primer design and 5′ RACE approach to generate chimeric mAbs, while avoiding the amplification of non-functional antibody genes from aberrant mRNA transcripts of the parental myeloma cell line (Strohal *et al.*, 1987; Irani *et al.*, 2008; Ding *et al.*, 2010). Although we eventually discovered that it was unnecessary to perform these techniques on a lambda light chain antibody, which is not known to harbour any non-functional genes, the method used should theoretically be applicable for immunoglobulin heavy and kappa light chain gene manipulations as well, and shall prove valuable for fuss-free generation of chimeric mouse-human antibodies for diagnostic use.

Although we have shown that the chimeric 5.2H7 mAb was successful in the detection of several CHIKV isolates, and can be stably and continuously expressed, there are concerns that the sensitivity of the COS-7L or CHO cell-expressed recombinant mAb was significantly lower than that of the

parental mAb, in the form of secreted hybridoma supernatant. However, it is worthwhile noting that due to the lack of optimisation of transfection efficiency and several other variables associated with recombinant mAb expression, the quantity of recombinantly-expressed chimeric 5.2H7 mAb cannot be compared to that of mAbs secreted by the original 5.2H7 parental hybridoma cell line. Theoretically, under optimised conditions, the production of the chimeric 5.2H7 mAb could be cost-effectively up-scaled, with the option of further downsteam processing such as purification and concentration. This will allow for much improved performance of the antibody, and increased sensitivity towards CHIKV antigens. In summary, we have produced a reagent that has practical potential for use in commercial diagnostics.

8.5. FINAL CONCLUSIONS

In summary, we have developed and evaluated reagents that may prove valuable as reagents for the detection of CHIKV, and perhaps play significant roles in future research of the virus. Based on samples tested, the assessment of the anti-E2 mAbs and recombinant proteins, produced both in this project and by our collaborators, has led to the creation of a sensitive and specific diagnostic assay for the detection of anti-CHIKV antibodies in human sera. In addition, we have showed the potential of the anti-E2 mAbs to specifically detect minute quantities of CHIKV antigen, and their use in detecting CHIKV in mosquito vectors. Furthermore, the CP-specific mAbs were shown to be reactive towards several alphaviruses, including all strains of CHIKV tested in this study. These mAbs showed excellent sensitivity in numerous assays including IHC, where small quantities of CHIKV protein were detected in murine feet tissue weeks post-infection, highlighting the persistence of CHIKV in the mouse model. Through the process of mapping the binding sites of these CP-specific mAbs, we have also confirmed the presence of sCP and revealed that the C-terminus of the CP may be required for antibody recognition of the protein. Finally, we have successfully created a chimeric mouse-human mAb for use in serological diagnostics and shown that the recombinant mAb retained the ability of the parent mAb to recognize different CHIKV genotypes. Future efforts to stably express the chimeric 5.2H7 antibody will provide a practical alternative to the use of human reference sera in commercial diagnostic test kits.

BIBLIOGRAPHY LIST

Adesina, O. A. and Odelola, H. A. (1991). Ecological distribution of Chikungunya haemagglutination inhibition antibodies in human and domestic animals in Nigeria. *Tropical and Geographical Medicine* **43**(3): 271-275.

Aguilar, P. V., Weaver, S. C. and Basler, C. F. (2007). Capsid protein of eastern equine encephalitis virus inhibits host cell gene expression. *Journal of Virology* **81**(8): 3866-3876.

Ahmed, N. H. and Broor, S. (2014). Comparison of NS1 antigen detection ELISA, real time RT-PCR and virus isolation for rapid diagnosis of dengue infection in acute phase. *Journal of Vector Borne Diseases* **51**(3): 194-199.

Akahata, W. and Nabel, G. J. (2012). A specific domain of the chikungunya virus e2 protein regulates particle formation in human cells: implications for alphavirus vaccine design. *Journal of Virology* **86**(16): 8879-8883.

Akahata, W., Yang, Z. Y., Andersen, H., Sun, S., Holdaway, H. A., Kong, W. P., Lewis, M. G., Higgs, S., Rossmann, M. G., Rao, S. and Nabel, G. J. (2010). A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. *Nature Medicine* **16**(3): 334-338.

An, Z. (2010). Monoclonal antibodies - a proven and rapidly expanding therapeutic modality for human diseases. *Protein and Cell* **1**(4): 319-330.

Anderson, N. W., Jespersen, D. J., Rollins, L., Seaton, B., Prince, H. E. and Theel, E. S. (2014). Detection of the dengue virus NS1 antigen using an enzyme immunoassay. *Diagnostic Microbiology and Infectious Disease* **79**(2): 194-197.

Angelini, P., Macini, P., Finarelli, A. C., Pol, C., Venturelli, C., Bellini, R. and Dottori, M. (2008). Chikungunya epidemic outbreak in Emilia-Romagna (Italy) during summer 2007. *Parassitologia* **50**(1-2): 97-98.

Angelini, R., Finarelli, A. C., Angelini, P., Po, C., Petropulacos, K., Macini, P., Fiorentini, C., Fortuna, C., Venturi, G., Romi, R., Majori, G., Nicoletti, L., Rezza, G. and Cassone, A. (2007). An outbreak of chikungunya fever in the province of Ravenna, Italy. *Euro Surveillance* **12**(9): E070906 070901.

Attallah, A. M., Ismail, H., Tabll, A. A., Shiha, G. E. and El-Dosoky, I. (2003). A novel antigen detection immunoassay for field diagnosis of hepatitis C virus infection. *Journal of Immunoassay and Immunochemistry* **24**(4): 395-407.

Baba, M., Logue, C. H., Oderinde, B., Abdulmaleek, H., Williams, J., Lewis, J., Laws, T. R., Hewson, R., Marcello, A. and P, D. A. (2013). Evidence of arbovirus co-infection in suspected febrile malaria and typhoid patients in Nigeria. *J Infect Dev Ctries* **7**(1): 51-59.

Bell, J. R., Bond, M. W., Hunkapiller, M. W., Strauss, E. G., Strauss, J. H., Yamamoto, K. and Simizu, B. (1983). Structural proteins of Western equine encephalitis virus: amino acid compositions and N-terminal sequences. *Journal of Virology* **45**(2): 708-714.

Bessaud, M., Peyrefitte, C. N., Pastorino, B. A., Tock, F., Merle, O., Colpart, J. J., Dehecq, J. S., Girod, R., Jaffar-Bandjee, M. C., Glass, P. J., Parker, M., Tolou, H. J. and Grandadam, M. (2006). Chikungunya virus strains, Reunion Island outbreak. *Emerging Infectious Diseases* **12**(10): 1604-1606.

Black, R. E., Cousens, S., Johnson, H. L., Lawn, J. E., Rudan, I., Bassani, D. G., Jha, P., Campbell, H., Walker, C. F., Cibulskis, R., Eisele, T., Liu, L., Mathers, C., Child Health Epidemiology Reference Group of, W. H. O. and Unicef (2010). Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* **375**(9730): 1969-1987.

Blacksell, S. D., Tanganuchitcharnchai, A., Jarman, R. G., Gibbons, R. V., Paris, D. H., Bailey, M. S., Day, N. P., Premaratna, R., Lalloo, D. G. and de Silva, H. J. (2011). Poor diagnostic accuracy of commercial antibody-based assays for the diagnosis of acute Chikungunya infection. *Clinical and Vaccine Immunology* **18**(10): 1773-1775.

Blitvich, B. J., Bowen, R. A., Marlenee, N. L., Hall, R. A., Bunning, M. L. and Beaty, B. J. (2003). Epitope-blocking enzyme-linked immunosorbent assays for detection of west nile virus antibodies in domestic mammals. *Journal of Clinical Microbiology* **41**(6): 2676-2679.

Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S. and Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* **4**(6): 1633-1649.

Borgherini, G., Poubeau, P., Jossaume, A., Gouix, A., Cotte, L., Michault, A., Arvin-Berod, C. and Paganin, F. (2008). Persistent arthralgia associated with chikungunya virus: a study of 88 adult patients on reunion island. *Clinical Infectious Diseases* **47**(4): 469-475.

Borgherini, G., Poubeau, P., Staikowsky, F., Lory, M., Le Moullec, N., Becquart, J. P., Wengling, C., Michault, A. and Paganin, F. (2007). Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients. *Clin Infect Dis* **44**(11): 1401-1407.

Both, L., Banyard, A. C., van Dolleweerd, C., Wright, E., Ma, J. K. and Fooks, A. R. (2013). Monoclonal antibodies for prophylactic and therapeutic use against viral infections. *Vaccine* **31**(12): 1553-1559.

Brandler, S., Ruffie, C., Combredet, C., Brault, J. B., Najburg, V., Prevost, M. C., Habel, A., Tauber, E., Despres, P. and Tangy, F. (2013). A recombinant measles vaccine expressing chikungunya virus-like particles is strongly immunogenic and protects mice from lethal challenge with chikungunya virus. *Vaccine*.

Brighton, S. W., Prozesky, O. W. and de la Harpe, A. L. (1983). Chikungunya virus infection. A retrospective study of 107 cases. *South African Medical Journal* **63**(9): 313-315.

Broom, A. K., Hall, R. A., Johansen, C. A., Oliveira, N., Howard, M. A., Lindsay, M. D., Kay, B. H. and Mackenzie, J. S. (1998). Identification of Australian arboviruses in inoculated cell cultures using monoclonal antibodies in ELISA. *Pathology* **30**(3): 286-288.

Burke, D. and Keegstra, K. (1979). Carbohydrate structure of Sindbis virus glycoprotein E2 from virus grown in hamster and chicken cells. *Journal of Virology* **29**(2): 546-554.

By, Y., Durand-Gorde, J. M., Condo, J., Lejeune, P. J., Mallet, B., Carayon, P., Guieu, R. and Ruf, J. (2009). Production of an agonist-like monoclonal antibody to the human A2A receptor of adenosine for clinical use. *Molecular Immunology* **46**(3): 400-405.

Caglioti, C., Lalle, E., Castilletti, C., Carletti, F., Capobianchi, M. R. and Bordi, L. (2013). Chikungunya virus infection: an overview. *New Microbiologica* **36**(3): 211-227.

Carey, D. E. (1971). Chikungunya and dengue: a case of mistaken identity? *Journal of the History of Medicine and Allied Sciences* **26**(3): 243-262.

Carey, D. E., Myers, R. M., DeRanitz, C. M., Jadhav, M. and Reuben, R. (1969). The 1964 chikungunya epidemic at Vellore, South India, including observations on concurrent Dengue. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **63**(4): 434-445.

Carroll, W. L., Mendel, E. and Levy, S. (1988). Hybridoma fusion cell lines contain an aberrant kappa transcript. *Molecular Immunology* **25**(10): 991-995.

Chandak, N. H., Kashyap, R. S., Kabra, D., Karandikar, P., Saha, S. S., Morey, S. H., Purohit, H. J., Taori, G. M. and Daginawala, H. F. (2009). Neurological complications of Chikungunya virus infection. *Neurology India* **57**(2): 177-180.

Cho, B., Kim, J., Cho, J. E., Jeon, B. Y. and Park, S. (2008). Expression of the capsid protein of Chikungunya virus in a baculovirus for serodiagnosis of Chikungunya disease. *Journal of Virological Methods* **154**(1-2): 154-159.

Choi, H. K., Lee, S., Zhang, Y. P., McKinney, B. R., Wengler, G., Rossmann, M. G. and Kuhn, R. J. (1996). Structural analysis of Sindbis virus capsid mutants involving assembly and catalysis. *Journal of Molecular Biology* **262**(2): 151-167.

Choi, H. K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M. G. and Wengler, G. (1991). Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. *Nature* **354**(6348): 37-43.

Chow, A., Her, Z., Ong, E. K., Chen, J. M., Dimatatac, F., Kwek, D. J., Barkham, T., Yang, H., Renia, L., Leo, Y. S. and Ng, L. F. (2011). Persistent arthralgia induced by Chikungunya virus

infection is associated with interleukin-6 and granulocyte macrophage colony-stimulating factor. *Journal of Infectious Diseases* **203**(2): 149-157.

Chretien, J. P. and Linthicum, K. J. (2007). Chikungunya in Europe: what's next? *Lancet* **370**(9602): 1805-1806.

Clancy, P. (1991). Production and Application of Monoclonal Antibodies to the Australian Alphaviruses. *MSc Thesis. School of Tropical Veterinary Science and Agriculture, James Cook University of North Queensland, Townsville, Queensland, Australia*.

Clark, D. C., Lobigs, M., Lee, E., Howard, M. J., Clark, K., Blitvich, B. J. and Hall, R. A. (2007). In situ reactions of monoclonal antibodies with a viable mutant of Murray Valley encephalitis virus reveal an absence of dimeric NS1 protein. *Journal of General Virology* **88**(4): 1175-1183.

Coleman, R. E., Hochberg, L. P., Putnam, J. L., Swanson, K. I., Lee, J. S., McAvin, J. C., Chan, A. S., Oguinn, M. L., Ryan, J. R., Wirtz, R. A., Moulton, J. K., Dave, K. and Faulde, M. K. (2009). Use of vector diagnostics during military deployments: recent experience in Iraq and Afghanistan. *Military Medicine* **174**(9): 904-920.

Couderc, T., Khandoudi, N., Grandadam, M., Visse, C., Gangneux, N., Bagot, S., Prost, J. F. and Lecuit, M. (2009). Prophylaxis and therapy for Chikungunya virus infection. *Journal of Infectious Diseases* **200**(4): 516-523.

da Costa, V. G., Marques-Silva, A. C. and Moreli, M. L. (2014). A meta-analysis of the diagnostic accuracy of two commercial NS1 antigen ELISA tests for early dengue virus detection. *PLoS ONE* **9**(4): e94655.

Davis, N. L., Pence, D. F., Meyer, W. J., Schmaljohn, A. L. and Johnston, R. E. (1987). Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein. *Virology* **161**(1): 101-108.

Diallo, M., Thonnon, J., Traore-Lamizana, M. and Fontenille, D. (1999). Vectors of Chikungunya virus in Senegal: current data and transmission cycles. *American Journal of Tropical Medicine and Hygiene* **60**(2): 281-286.

Ding, G., Chen, X., Zhu, J. and Cao, B. (2010). Identification of two aberrant transcripts derived from a hybridoma with amplification of functional immunoglobulin variable genes. *Cellular and Molecular Immunology* **7**(5): 349-354.

Diseases, N. I. o. A. a. I. (2011). NIAID Category A, B, and C priority pathogens, National Institute of Allergy and Infectious Diseases [online], http://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx.

Doucoure, S., Mouchet, F., Cornelie, S., Drame, P. M., D'Ortenzio, E., Dehecq, J. S. and Remoue, F. (2014). Human Antibody Response to Aedes albopictus Salivary Proteins: A Potential

Biomarker to Evaluate the Efficacy of Vector Control in an Area of Chikungunya and Dengue Virus Transmission. *Biomed Res Int* **2014**: 746509.

Duan, L. and Pomerantz, R. J. (1994). Elimination of endogenous aberrant kappa chain transcripts from sp2/0-derived hybridoma cells by specific ribozyme cleavage: utility in genetic therapy of HIV-1 infections. *Nucleic Acids Research* **22**(24): 5433-5438.

Economopoulou, A., Dominguez, M., Helynck, B., Sissoko, D., Wichmann, O., Quenel, P., Germonneau, P. and Quatresous, I. (2009). Atypical Chikungunya virus infections: clinical manifestations, mortality and risk factors for severe disease during the 2005-2006 outbreak on Reunion. *Epidemiology and Infection* **137**(4): 534-541.

Edwards, C. J., Welch, S. R., Chamberlain, J., Hewson, R., Tolley, H., Cane, P. A. and Lloyd, G. (2007). Molecular diagnosis and analysis of Chikungunya virus. *Journal of Clinical Virology* **39**(4): 271-275.

Elgizoli, M., Dai, Y., Kempf, C., Koblet, H. and Michel, M. R. (1989). Semliki Forest virus capsid protein acts as a pleiotropic regulator of host cellular protein synthesis. *Journal of Virology* **63**(7): 2921-2928.

Enserink, M. (2006). Infectious diseases. Massive outbreak draws fresh attention to little-known virus. *Science* **311**(5764): 1085.

Ernould, S., Walters, H., Alessandri, J. L., Llanas, B., Jaffar, M. C., Robin, S., Attali, T., Ramful, D. and Combes, J. C. (2008). [Chikungunya in paediatrics: Epidemic of 2005-2006 in Saint-Denis, Reunion Island.]. *Archives de Pediatrie*.

Ezzedine, K., Cazanave, C., Pistone, T., Receveur, M. C., Neau, D., Ragnaud, J. M. and Malvy, D. (2008). Dual infection by chikungunya virus and other imported infectious agent in a traveller returning from India. *Travel Medicine and Infectious Disease* **6**(3): 152-154.

Favre, D., Studer, E. and Michel, M. R. (1994). Two nucleolar targeting signals present in the N-terminal part of Semliki Forest virus capsid protein. *Archives of Virology* **137**(1-2): 149-155.

Fernandez, D., Valle, I., Llamos, R., Guerra, M., Sorell, L. and Gavilondo, J. (1994). Rapid detection of rotavirus in faeces using a dipstick system with monoclonal antibodies and colloidal gold as marker. *Journal of Virological Methods* **48**(2-3): 315-323.

Firth, A. E., Chung, B. Y., Fleeton, M. N. and Atkins, J. F. (2008). Discovery of frameshifting in Alphavirus 6K resolves a 20-year enigma. *Virology Journal* **5**: 108.

Fourie, E. D. and Morrison, J. G. (1979). Rheumatoid arthritic syndrome after Chikungunya fever. *South African Medical Journal* **56**(4): 130-132.

Frank, C., Schoneberg, I. and Stark, K. (2011). Trends in imported chikungunya virus infections in Germany, 2006-2009. *Vector Borne and Zoonotic Diseases* **11**(6): 631-636.

Fric, J., Bertin-Maghit, S., Wang, C. I., Nardin, A. and Warter, L. (2012). Treatment of Chikungunya virus infection using human monoclonal antibodies. *Journal of Infectious Diseases*.

Gardner, J., Anraku, I., Le, T. T., Larcher, T., Major, L., Roques, P., Schroder, W. A., Higgs, S. and Suhrbier, A. (2010). Chikungunya virus arthritis in adult wild-type mice. *Journal of Virology* **84**(16): 8021-8032.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E. and Frolov, I. (2007). The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *Journal of Virology* **81**(5): 2472-2484.

Gasque, P., Couderc, T., Lecuit, M., Roques, P. and Ng, L. F. (2015). Chikungunya virus pathogenesis and immunity. *Vector Borne and Zoonotic Diseases* **15**(4): 241-249.

Gerardin, P., Barau, G., Michault, A., Bintner, M., Randrianaivo, H., Choker, G., Lenglet, Y., Touret, Y., Bouveret, A., Grivard, P., Roux, K. L., Blanc, S., Schuffenecker, I., Couderc, T., Arenzana-Seisdedos, F., Lecuit, M. and Robillard, P. Y. (2008). Multidisciplinary prospective study of mother-to-child Chikungunya virus infections on the island of La Reunion. *PLoS Medicine* **5**(3): e60.

Gibney, K. B., Fischer, M., Prince, H. E., Kramer, L. D., St George, K., Kosoy, O. L., Laven, J. J. and Staples, J. E. (2011). Chikungunya fever in the United States: a fifteen year review of cases. *Clinical Infectious Diseases* **52**(5): e121-126.

Glenney, J. R., Jr., Zokas, L. and Kamps, M. P. (1988). Monoclonal antibodies to phosphotyrosine. *Journal of Immunological Methods* **109**(2): 277-285.

Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Pyke, A. T., Suhrbier, A. and Hall, R. A. (2013). Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model. *Clinical Immunology* **149**(3): 487-497.

Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Suhrbier, A. and Hall, R. A. (2014). Monoclonal antibodies specific for the capsid protein of chikungunya virus suitable for multiple applications. *Journal of General Virology*.

Goh, L. Y. H., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Suhrbier, A. and Hall, R. A. (in press). Monoclonal antibodies specific for the capsid protein of chikungunya virus suitable for multiple applications. *J Gen Virol*.

Grandadam, M., Caro, V., Plumet, S., Thiberge, J. M., Souares, Y., Failloux, A. B., Tolou, H. J., Budelot, M., Cosserat, D., Leparc-Goffart, I. and Despres, P. (2011). Chikungunya virus, southeastern France. *Emerging Infectious Diseases* **17**(5): 910-913.

Grandien, M. (1996). Viral diagnosis by antigen detection techniques. *Clinical and Diagnostic Virology* **5**(2-3): 81-90.

Griffin, D. E. (2007). Fields Virology, Chapter 31 - Alphaviruses, Lippincott Williams & Wilkins (LWW).

Hackett, J., Jr., Hoff-Velk, J., Golden, A., Brashear, J., Robinson, J., Rapp, M., Klass, M., Ostrow, D. H. and Mandecki, W. (1998). Recombinant mouse-human chimeric antibodies as calibrators in immunoassays that measure antibodies to Toxoplasma gondii. *Journal of Clinical Microbiology* **36**(5): 1277-1284.

Hall, R. A., Broom, A. K., Hartnett, A. C., Howard, M. J. and Mackenzie, J. S. (1995). Immunodominant epitopes on the NS1 protein of MVE and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum. *Journal of Virological Methods* **51**(2-3): 201-210.

Hall, R. A., Burgess, G. W. and Kay, B. H. (1988). Type-specific monoclonal antibodies produced to proteins of Murray Valley encephalitis virus. *Immunology and Cell Biology* **66**(1): 51-56.

Hall, R. A., Tan, S. E., Selisko, B., Slade, R., Hobson-Peters, J., Canard, B., Hughes, M., Leung, J. Y., Balmori-Melian, E., Hall-Mendelin, S., Pham, K. B., Clark, D. C., Prow, N. A. and Khromykh, A. A. (2009). Monoclonal antibodies to the West Nile virus NS5 protein map to linear and conformational epitopes in the methyltransferase and polymerase domains. *Journal of General Virology* **90**(Pt 12): 2912-2922.

Halstead, S. B. (2015). Reappearance of chikungunya, formerly called dengue, in the americas. *Emerging Infectious Diseases* **21**(4).

Hassing, R. J., Heijstek, M. W., van Beek, Y., van Doornum, G. J. and Overbosch, D. (2008). [First case of chikungunya diagnosed in the Netherlands]. *Nederlands Tijdschrift voor Geneeskunde* **152**(2): 101-103.

Hastie, M. L., Headlam, M. J., Patel, N. B., Bukreyev, A. A., Buchholz, U. J., Dave, K. A., Norris, E. L., Wright, C. L., Spann, K. M., Collins, P. L. and Gorman, J. J. (2012). The human respiratory syncytial virus nonstructural protein 1 regulates type I and type II interferon pathways. *Molecular and Cellular Proteomics* **11**(5): 108-127.

Hearn, H. J., Jr. and Rainey, C. T. (1963). Cross-protection in animals infected with group a arboviruses. *Journal of Immunology* **90**: 720-724.

Higgs, S. (2014). Chikungunya virus: a major emerging threat. *Vector Borne and Zoonotic Diseases* **14**(8): 535-536.

Hoarau, J. J., Jaffar Bandjee, M. C., Krejbich Trotot, P., Das, T., Li-Pat-Yuen, G., Dassa, B., Denizot, M., Guichard, E., Ribera, A., Henni, T., Tallet, F., Moiton, M. P., Gauzere, B. A., Bruniquet, S., Jaffar Bandjee, Z., Morbidelli, P., Martigny, G., Jolivet, M., Gay, F., Grandadam, M., Tolou, H., Vieillard, V., Debre, P., Autran, B. and Gasque, P. (2010). Persistent chronic inflammation and infection by Chikungunya arthritogenic alphavirus in spite of a robust host immune response. *Journal of Immunology* **184**(10): 5914-5927.

Hobson-Peters, J., Toye, P., Sanchez, M. D., Bossart, K. N., Wang, L. F., Clark, D. C., Cheah, W. Y. and Hall, R. A. (2008). A glycosylated peptide in the West Nile virus envelope protein is immunogenic during equine infection. *Journal of General Virology* **89**(Pt 12): 3063-3072.

Hommel, D., Heraud, J. M., Hulin, A. and Talarmin, A. (2000). Association of Tonate virus (subtype IIIB of the Venezuelan equine encephalitis complex) with encephalitis in a human. *Clinical Infectious Diseases* **30**(1): 188-190.

Hong, E. M., Perera, R. and Kuhn, R. J. (2006). Alphavirus capsid protein helix I controls a checkpoint in nucleocapsid core assembly. *Journal of Virology* **80**(18): 8848-8855.

Hopkins, R. and Esposito, D. (2009). A rapid method for titrating baculovirus stocks using the Sf-9 Easy Titer cell line. *Biotechniques* **47**(3): 785-788.

Horwood, P., Bande, G., Dagina, R., Guillaumot, L., Aaskov, J. and Pavlin, B. (2013). The threat of chikungunya in Oceania. *Western Pac Surveill Response J* **4**(2): 8-10.

Hunt, A. R., Hall, R. A., Kerst, A. J., Nasci, R. S., Savage, H. M., Panella, N. A., Gottfried, K. L., Burkhalter, K. L. and Roehrig, J. T. (2002). Detection of West Nile virus antigen in mosquitoes and avian tissues by a monoclonal antibody-based capture enzyme immunoassay. *Journal of Clinical Microbiology* **40**(6): 2023-2030.

Ichikawa, K., Tsutsumi, A., Atsumi, T., Matsuura, E., Kobayashi, S., Hughes, G. R., Khamashta, M. A. and Koike, T. (1999). A chimeric antibody with the human gamma1 constant region as a putative standard for assays to detect IgG beta2-glycoprotein I-dependent anticardiolipin and anti-beta2-glycoprotein I antibodies. *Arthritis and Rheumatism* **42**(11): 2461-2470.

Irani, Y., Tea, M., Tilton, R. G., Coster, D. J., Williams, K. A. and Brereton, H. M. (2008). PCR amplification of the functional immunoglobulin heavy chain variable gene from a hybridoma in the presence of two aberrant transcripts. *Journal of Immunological Methods* **336**(2): 246-250.

Ishida, I. and Simizu, B. (1981). Evidence for the presence of the minor capsid protein of Western equine encephalitis virus. *Archives of Virology* **67**(2): 159-164.

Jacobson, R. H. (1998). Validation of serological assays for diagnosis of infectious diseases. *Revue Scientifique et Technique, Office International des Epizooties* **17**(2): 469-526.

Jadhav, M., Namboodripad, M., Carman, R. H., Carey, D. E. and Myers, R. M. (1965). Chikungunya disease in infants and children in Vellore: a report of clinical and haematological features of virologically proved cases. *Indian Journal of Medical Research* **53**(8): 764-776.

Jaffar-Bandjee, M. C., Ramful, D., Gauzere, B. A., Hoarau, J. J., Krejbich-Trotot, P., Robin, S., Ribera, A., Selambarom, J. and Gasque, P. (2010). Emergence and clinical insights into the pathology of Chikungunya virus infection. *Expert Review of Anti-Infective Therapy* **8**(9): 987-996.

Jain, M., Rai, S. and Chakravarti, A. (2008). Chikungunya: a review. *Tropical Doctor* **38**(2): 70-72.

Jakob, R. (1994). Nucleolar accumulation of Semliki Forest virus nucleocapsid C protein: influence of metabolic status, cytoskeleton and receptors. *Journal of Medical Microbiology* **40**(6): 389-392.

Jones, M. L. and Barnard, R. T. (2005). Chimerization of multiple antibody classes using splice overlap extension PCR. *Biotechniques* **38**(2): 181-182.

Jones, M. L. and Barnard, R. T. (2007). Use of chimeric antibodies as positive controls in an enzyme-linked immunosorbent assay for diagnosis of scrub typhus (infection by Orientia tsutsugamushi). *Clinical and Vaccine Immunology* **14**(10): 1307-1310.

Jose, J., Przybyla, L., Edwards, T. J., Perera, R., Burgner, J. W., 2nd and Kuhn, R. J. (2013). Interactions of the cytoplasmic domain of Sindbis virus E2 with nucleocapsid cores promote alphavirus budding. *Journal of Virology* **86**(5): 2585-2599.

Jose, J., Snyder, J. E. and Kuhn, R. J. (2009). A structural and functional perspective of alphavirus replication and assembly. *Future Microbiology* **4**: 837-856.

Joshi, R., Colford, J. M., Jr., Reingold, A. L. and Kalantri, S. (2008). Nonmalarial acute undifferentiated fever in a rural hospital in central India: diagnostic uncertainty and overtreatment with antimalarial agents. *American Journal of Tropical Medicine and Hygiene* **78**(3): 393-399.

Josseran, L., Paquet, C., Zehgnoun, A., Caillere, N., Le Tertre, A., Solet, J. L. and Ledrans, M. (2006). Chikungunya disease outbreak, Reunion Island. *Emerging Infectious Diseases* **12**(12): 1994-1995.

Jupp, P. G. and McIntosh, B. M. (1990). Aedes furcifer and other mosquitoes as vectors of Chikungunya virus at Mica, northeastern Transvaal, South Africa. *Journal of the American Mosquito Control Association* **6**(3): 415-420.

Kam, Y. W., Lee, W. W., Simarmata, D., Harjanto, S., Teng, T. S., Tolou, H., Chow, A., Lin, R. T., Leo, Y. S., Renia, L. and Ng, L. F. (2012). Longitudinal analysis of the human antibody response to Chikungunya virus infection: implications for serodiagnosis and vaccine development. *Journal of Virology* **86**(23): 13005-13015.

Kappler, U. and Nouwens, A. S. (2013). The molybdoproteome of Starkeya novella--insights into the diversity and functions of molybdenum containing proteins in response to changing growth conditions. *Metallomics* **5**(4): 325-334.

Kashyap, R. S., Morey, S. H., Ramteke, S. S., Chandak, N. H., Parida, M., Deshpande, P. S., Purohit, H. J., Taori, G. M. and Daginawala, H. F. (2010). Diagnosis of Chikungunya fever in an Indian population by an indirect enzyme-linked immunosorbent assay protocol based on an antigen detection assay: a prospective cohort study. *Clinical and Vaccine Immunology* **17**(2): 291-297.

Kehoe, J. W., Velappan, N., Walbolt, M., Rasmussen, J., King, D., Lou, J., Knopp, K., Pavlik, P., Marks, J. D., Bertozzi, C. R. and Bradbury, A. R. (2006). Using phage display to select antibodies recognizing post-translational modifications independently of sequence context. *Molecular and Cellular Proteomics* **5**(12): 2350-2363.

Keller, A., Nesvizhskii, A. I., Kolker, E. and Aebersold, R. (2002). Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Analytical Chemistry* **74**(20): 5383-5392.

Khai Ming, C., Thain, S., Thaung, U., Tin, U., Myint, K. S., Swe, T., Halstead, S. B. and Diwan, A. R. (1974). Clinical and laboratory studies on haemorrhagic fever in Burma, 1970-72. *Bulletin of the World Health Organization* **51**(3): 227-235.

Kielian, M., Chanel-Vos, C. and Liao, M. (2010). Alphavirus Entry and Membrane Fusion. *Viruses* **2**(4): 796-825.

Kielian, M. and Rey, F. A. (2006). Virus membrane-fusion proteins: more than one way to make a hairpin. *Nature Reviews. Microbiology* **4**(1): 67-76.

Knight, R. L., Schultz, K. L., Kent, R. J., Venkatesan, M. and Griffin, D. E. (2009). Role of N-linked glycosylation for sindbis virus infection and replication in vertebrate and invertebrate systems. *Journal of Virology* **83**(11): 5640-5647.

Knope, K., Whelan, P., Smith, D., Johansen, C., Moran, R., Doggett, S., Sly, A., Hobby, M., Wright, P., Nicholson, J., National, A. and Malaria Advisory, C. (2013). Arboviral diseases and malaria in Australia, 2010-11: annual report of the National Arbovirus and Malaria Advisory Committee. *Commun Dis Intell Q Rep* **37**(1): E1-20.

Kohler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**(5517): 495-497.

Kosasih, H., Widjaja, S., Surya, E., Hadiwijaya, S. H., Butarbutar, D. P., Jaya, U. A., Nurhayati, Alisjahbana, B. and Williams, M. (2012). Evaluation of two IgM rapid immunochromatographic tests during circulation of Asian lineage Chikungunya virus. *Southeast Asian Journal of Tropical Medicine and Public Health* **43**(1): 55-61.

Kumar, K. J., Manjunath, V. G., Shailashree, M. and Girish, G. N. (2012). Coinfection with dengue and chikungunya--a case report. *Journal of the Indian Medical Association* **110**(10): 749, 752.

Kumar, N. P., Joseph, R., Kamaraj, T. and Jambulingam, P. (2008). A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India. *Journal of General Virology* **89**(8): 1945-1948.

Kutemeier, G., Harloff, C. and Mocikat, R. (1992). Rapid isolation of immunoglobulin variable genes from cell lysates of rat hybridomas by polymerase chain reaction. *Hybridoma* **11**(1): 23-32.

L'Vov D, K., Gromashevskii, V. L., Skvortsova, T. M., Berezina, L. K. and Zakarian, V. A. (1979). [Kyzylagach virus (family Togaviridae, genus alphaviruses), a new arbovirus isolated from Culex modestus mosquitoes trapped in the Azerbaijani SSR]. *Voprosy Virusologii*(5): 519-523.

Labadie, K., Larcher, T., Joubert, C., Mannioui, A., Delache, B., Brochard, P., Guigand, L., Dubreil, L., Lebon, P., Verrier, B., de Lamballerie, X., Suhrbier, A., Cherel, Y., Le Grand, R. and Roques, P. (2010). Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. *Journal of Clinical Investigation* **120**(3): 894-906.

Lam, S. K., Chua, K. B., Hooi, P. S., Rahimah, M. A., Kumari, S., Tharmaratnam, M., Chuah, S. K., Smith, D. W. and Sampson, I. A. (2001). Chikungunya infection--an emerging disease in Malaysia. *Southeast Asian Journal of Tropical Medicine and Public Health* **32**(3): 447-451.

Lambrechts, L., Scott, T. W. and Gubler, D. J. (2010). Consequences of the expanding global distribution of Aedes albopictus for dengue virus transmission. *PLoS Neglected Tropical Diseases* **4**(5): e646.

Lanciotti, R. S. and Valadere, A. M. (2014). Transcontinental movement of Asian genotype chikungunya virus. *Emerging Infectious Diseases* **20**(8): 1400-1402.

Laurent, P., Le Roux, K., Grivard, P., Bertil, G., Naze, F., Picard, M., Staikowsky, F., Barau, G., Schuffenecker, I. and Michault, A. (2007). Development of a sensitive real-time reverse transcriptase PCR assay with an internal control to detect and quantify chikungunya virus. *Clinical Chemistry* **53**(8): 1408-1414.

Lenglet, Y., Barau, G., Robillard, P. Y., Randrianaivo, H., Michault, A., Bouveret, A., Gerardin, P., Boumahni, B., Touret, Y., Kauffmann, E., Schuffenecker, I., Gabriele, M. and Fourmaintraux, A. (2006). [Chikungunya infection in pregnancy: Evidence for intrauterine infection in pregnant women and vertical transmission in the parturient. Survey of the Reunion Island outbreak]. *Journal de Gynecologie, Obstetrique et Biologie de la Reproduction* **35**(6): 578-583.

Li, G. and Rice, C. M. (1993). The signal for translational readthrough of a UGA codon in Sindbis virus RNA involves a single cytidine residue immediately downstream of the termination codon. *Journal of Virology* **67**(8): 5062-5067.

Little, M., Kipriyanov, S. M., Le Gall, F. and Moldenhauer, G. (2000). Of mice and men: hybridoma and recombinant antibodies. *Immunology Today* **21**(8): 364-370.

Lorono-Pino, M. A., Farfan-Ale, J. A., Blitvich, B. J., Beebe, J. L., Jarman, R. G. and Beaty, B. J. (2009). Evaluation of an epitope-blocking enzyme-linked immunosorbent assay for the diagnosis of West Nile virus infections in humans. *Clinical and Vaccine Immunology* **16**(5): 749-755.

Lumsden, W. H. (1955). An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. II. General description and epidemiology. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **49**(1): 33-57.

Majumdar, M., Singh, M. P., Pujhari, S. K., Bhatia, D., Chawla, Y. and Ratho, R. K. (2013). Hepatitis E virus antigen detection as an early diagnostic marker: report from India. *Journal of Medical Virology* **85**(5): 823-827.

Maltha, J., Guiraud, I., Lompo, P., Kabore, B., Gillet, P., Van Geet, C., Tinto, H. and Jacobs, J. (2014). Accuracy of PfHRP2 versus Pf-pLDH antigen detection by malaria rapid diagnostic tests in hospitalized children in a seasonal hyperendemic malaria transmission area in Burkina Faso. *Malaria Journal* **13**: 20.

Mansuy, J. M., Grouteau, E., Mengelle, C., Claudet, I. and Izopet, J. (2014). Chikungunya in the Caribbean--threat for Europe. *Emerging Infectious Diseases* **20**(8): 1423-1425.

Marsh, M. and Helenius, A. (2006). Virus entry: open sesame. *Cell* **124**(4): 729-740.

Mavalankar, D., Shastri, P., Bandyopadhyay, T., Parmar, J. and Ramani, K. V. (2008). Increased mortality rate associated with Chikungunya epidemic, Ahmedabad, India. *Emerging Infectious Diseases* **14**(3): 412-415.

McGill, P. E. (1995). Viral infections: alpha-viral arthropathy. *Bailliere's Clinical Rheumatology* **9**(1): 145-150.

McIntosh, B. M. and Jupp, P. G. (1970). Attempts to transmit Chikungunya virus with six species of mosquito. *Journal of Medical Entomology* **7**(5): 615-618.

Melancon, P. and Garoff, H. (1987). Processing of the Semliki Forest virus structural polyprotein: role of the capsid protease. *Journal of Virology* **61**(5): 1301-1309.

Metz, S. W., Gardner, J., Geertsema, C., Le, T. T., Goh, L., Vlak, J. M., Suhrbier, A. and Pijlman, G. P. (2013). Effective chikungunya virus-like particle vaccine produced in insect cells. *PLoS Neglected Tropical Diseases* **7**(3): e2124.

Metz, S. W., Geertsema, C., Martina, B. E., Andrade, P., Heldens, J. G., van Oers, M. M., Goldbach, R. W., Vlak, J. M. and Pijlman, G. P. (2011). Functional processing and secretion of Chikungunya virus E1 and E2 glycoproteins in insect cells. *Virology Journal* **8**: 353.

Miner, J. J., Aw-Yeang, H. X., Fox, J. M., Taffner, S., Malkova, O. N., Oh, S. T., Kim, A. H., Diamond, M. S., Lenschow, D. J. and Yokoyama, W. M. (2015). Chikungunya viral arthritis in the United States: A mimic of seronegative rheumatoid arthritis. *Arthritis Rheumatol*.

Mitchell, C. J., Miller, B. R. and Gubler, D. J. (1987). Vector competence of Aedes albopictus from Houston, Texas, for dengue serotypes 1 to 4, yellow fever and Ross River viruses. *Journal of the American Mosquito Control Association* **3**(3): 460-465.

Mizuno, Y., Kato, Y., Takeshita, N., Ujiie, M., Kobayashi, T., Kanagawa, S., Kudo, K., Lim, C. K. and Takasaki, T. (2011). Clinical and radiological features of imported chikungunya fever in Japan: a study of six cases at the National Center for Global Health and Medicine. *J Infect Chemother* **17**(3): 419-423.

Mohan, A. (2006). Chikungunya fever: clinical manifestations & management. *Indian Journal of Medical Research* **124**(5): 471-474.

Morrison, S. L. (2002). Cloning, expression, and modification of antibody V regions. *Curr Protoc Immunol* **Chapter 2**: Unit 2 12.

Morrison, S. L., Johnson, M. J., Herzenberg, L. A. and Oi, V. T. (1984). Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proceedings of the National Academy of Sciences of the United States of America* **81**(21): 6851-6855.

Mukhopadhyay, S., Zhang, W., Gabler, S., Chipman, P. R., Strauss, E. G., Strauss, J. H., Baker, T. S., Kuhn, R. J. and Rossmann, M. G. (2006). Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. *Structure* **14**(1): 63-73.

Munasinghe, D. R., Amarasekera, P. J. and Fernando, C. F. (1966). An epidemic of dengue-like fever in Ceylon (chikungunya--a clinical and haematological study. *Ceylon Medical Journal* **11**(4): 129-142.

Nasci, R. S., Gottfried, K. L., Burkhalter, K. L., Ryan, J. R., Emmerich, E. and Dave, K. (2003). Sensitivity of the VecTest antigen assay for eastern equine encephalitis and western equine encephalitis viruses. *Journal of the American Mosquito Control Association* **19**(4): 440-444.

Naze, F., Le Roux, K., Schuffenecker, I., Zeller, H., Staikowsky, F., Grivard, P., Michault, A. and Laurent, P. (2009). Simultaneous detection and quantitation of Chikungunya, dengue and West Nile viruses by multiplex RT-PCR assays and dengue virus typing using high resolution melting. *Journal of Virological Methods* **162**(1-2): 1-7.

Ng, L. C. and Hapuarachchi, H. C. (2010). Tracing the path of Chikungunya virus--evolution and adaptation. *Infection, Genetics and Evolution* **10**(7): 876-885.

Niedrig, M., Zeller, H., Schuffenecker, I., Drosten, C., Emmerich, P., Rumer, L. and Donoso-Mantke, O. (2009). International diagnostic accuracy study for the serological detection of chikungunya virus infection. *Clinical Microbiology and Infection* **15**(9): 880-884.

Nigg, E. A., Baeuerle, P. A. and Luhrmann, R. (1991). Nuclear import-export: in search of signals and mechanisms. *Cell* **66**(1): 15-22.

Nimmannitya, S., Halstead, S. B., Cohen, S. N. and Margiotta, M. R. (1969). Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. *American Journal of Tropical Medicine and Hygiene* **18**(6): 954-971.

Nitatpattana, N., Kanjanopas, K., Yoksan, S., Satimai, W., Vongba, N., Langdatsuwan, S., Nakgoi, K., Ratchakum, S., Wauquier, N., Souris, M., Auewarakul, P. and Gonzalez, J. P. (2014). Long-term persistence of Chikungunya virus neutralizing antibodies in human populations of North Eastern Thailand. *Virology Journal* **11**: 183.

Nossal, G. J. (1992). The molecular and cellular basis of affinity maturation in the antibody response. *Cell* **68**(1): 1-2.

Olaleye, O. D., Oladosu, L. A., Omilabu, S. A., Baba, S. S. and Fagbami, A. H. (1989). Complement fixing antibodies against arboviruses in horses at Lagos, Nigeria. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux* **42**(3): 321-325.

Olaleye, O. D., Omilabu, S. A. and Fagbami, A. H. (1988). Igbo-Ora virus (an alphavirus isolated in Nigeria): a serological survey for haemagglutination inhibiting antibody in humans and domestic animals. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **82**(6): 905-906.

Oliveira, N. M., Broom, A. K., Mackenzie, J. S., Smith, D. W., Lindsay, M. D., Kay, B. H. and Hall, R. A. (2006). Epitope-blocking enzyme-linked immunosorbent assay for detection of antibodies to Ross River virus in vertebrate sera. *Clinical and Vaccine Immunology* **13**(7): 814-817.

Organization, P.-A. H. (2011). Preparedness and Response for Chikungunya Virus: Introduction in the Americas. *Pan American Health Organization. Washington, D.C.* **ISBN: 978-92-75-11632-6**.

Ostermeier, C. and Michel, H. (1996). Improved cloning of antibody variable regions from hybridomas by an antisense-directed RNase H digestion of the P3-X63-Ag8.653 derived pseudogene mRNA. *Nucleic Acids Research* **24**(10): 1979-1980.

Owen, K. E. and Kuhn, R. J. (1996). Identification of a region in the Sindbis virus nucleocapsid protein that is involved in specificity of RNA encapsidation. *Journal of Virology* **70**(5): 2757-2763.

Owen, K. E. and Kuhn, R. J. (1997). Alphavirus budding is dependent on the interaction between the nucleocapsid and hydrophobic amino acids on the cytoplasmic domain of the E2 envelope glycoprotein. *Virology* **230**(2): 187-196.

Pal, P., Dowd, K. A., Brien, J. D., Edeling, M. A., Gorlatov, S., Johnson, S., Lee, I., Akahata, W., Nabel, G. J., Richter, M. K., Smit, J. M., Fremont, D. H., Pierson, T. C., Heise, M. T. and Diamond, M. S. (2013). Development of a highly protective combination monoclonal antibody therapy against Chikungunya virus. *PLoS Pathogens* **9**(4): e1003312.

Pialoux, G., Gauzere, B. A., Jaureguiberry, S. and Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. *Lancet Infectious Diseases* **7**(5): 319-327.

Pletnev, S. V., Zhang, W., Mukhopadhyay, S., Fisher, B. R., Hernandez, R., Brown, D. T., Baker, T. S., Rossmann, M. G. and Kuhn, R. J. (2001). Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. *Cell* **105**(1): 127-136.

Powers, A. M. (2011). Genomic evolution and phenotypic distinctions of Chikungunya viruses causing the Indian Ocean outbreak. *Exp Biol Med (Maywood)* **236**(8): 909-914.

Powers, A. M. (2014). Chikungunya virus control: is a vaccine on the horizon? *Lancet*.

Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., Strauss, J. H. and Weaver, S. C. (2001). Evolutionary relationships and systematics of the alphaviruses. *Journal of Virology* **75**(21): 10118-10131.

Powers, A. M. and Logue, C. H. (2007). Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *Journal of General Virology* **88**(Pt 9): 2363-2377.

Prabakaran, M., Ho, H. T., Prabhu, N., Velumani, S., Szyporta, M., He, F., Chan, K. P., Chen, L. M., Matsuoka, Y., Donis, R. O. and Kwang, J. (2009). Development of epitope-blocking ELISA for universal detection of antibodies to human H5N1 influenza viruses. *PLoS ONE* **4**(2): e4566.

Prow, T. W., Chen, X., Prow, N. A., Fernando, G. J., Tan, C. S., Raphael, A. P., Chang, D., Ruutu, M. P., Jenkins, D. W., Pyke, A., Crichton, M. L., Raphaelli, K., Goh, L. Y., Frazer, I. H., Roberts, M. S., Gardner, J., Khromykh, A. A., Suhrbier, A., Hall, R. A. and Kendall, M. A. (2010). Nanopatch-Targeted Skin Vaccination against West Nile Virus and Chikungunya Virus in Mice. *Small* **6**(16): 1776-1784.

Queyriaux, B., Armengaud, A., Jeannin, C., Couturier, E. and Peloux-Petiot, F. (2008). Chikungunya in Europe. *Lancet* **371**(9614): 723-724.

Ramful, D., Carbonnier, M., Pasquet, M., Bouhmani, B., Ghazouani, J., Noormahomed, T., Beullier, G., Attali, T., Samperiz, S., Fourmaintraux, A. and Alessandri, J. L. (2007). Mother-to-child transmission of Chikungunya virus infection. *Pediatric Infectious Disease Journal* **26**(9): 811-815.

Rampal, Sharda, M. and Meena, H. (2007). Neurological complications in Chikungunya fever. *Journal of the Association of Physicians of India* **55**: 765-769.

Ravi, V. (2006). Re-emergence of Chikungunya virus in India. *Indian J Med Microbiol* **24**(2): 83-84.

Reddy, V., Ravi, V., Desai, A., Parida, M., Powers, A. M. and Johnson, B. W. (2012). Utility of IgM ELISA, TaqMan real-time PCR, reverse transcription PCR, and RT-LAMP assay for the diagnosis of Chikungunya fever. *Journal of Medical Virology* **84**(11): 1771-1778.

Reichert, J. M. (2011). Antibody-based therapeutics to watch in 2011. *mAbs* **3**(1): 76-99.

Renault, P., Solet, J. L., Sissoko, D., Balleydier, E., Larrieu, S., Filleul, L., Lassalle, C., Thiria, J., Rachou, E., de Valk, H., Ilef, D., Ledrans, M., Quatresous, I., Quenel, P. and Pierre, V. (2007). A major epidemic of chikungunya virus infection on Reunion Island, France, 2005-2006. *American Journal of Tropical Medicine and Hygiene* **77**(4): 727-731.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S., Magurano, F., Silvi, G., Angelini, P., Dottori, M., Ciufolini, M. G., Majori, G. C. and Cassone, A. (2007). Infection with Chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* **370**(9602): 1840-1846.

Rianthavorn, P., Wuttirattanakowit, N., Prianantathavorn, K., Limpaphayom, N., Theamboonlers, A. and Poovorawan, Y. (2010). Evaluation of a rapid assay for detection of IgM antibodies to chikungunya. *Southeast Asian Journal of Tropical Medicine and Public Health* **41**(1): 92-96.

Rice, C. M. and Strauss, J. H. (1981). Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins. *Proceedings of the National Academy of Sciences of the United States of America* **78**(4): 2062-2066.

Robillard, P. Y., Boumahni, B., Gerardin, P., Michault, A., Fourmaintraux, A., Schuffenecker, I., Carbonnier, M., Djemili, S., Choker, G., Roge-Wolter, M. and Barau, G. (2006). [Vertical maternal fetal transmission of the chikungunya virus. Ten cases among 84 pregnant women]. *Presse Medicale* **35**(5 Pt 1): 785-788.

Robin, S., Ramful, D., Le Seach, F., Jaffar-Bandjee, M. C., Rigou, G. and Alessandri, J. L. (2008). Neurologic manifestations of pediatric chikungunya infection. *Journal of Child Neurology* **23**(9): 1028-1035.

Robinson, M. C. (1955). An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952-1953. I. Clinical features. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **49**: 28-32.

Ross, R. W. (1956). The Newala epidemic. III. The virus: isolation, pathogenic properties and relationship to the epidemic. *Journal of Hygiene* **54**: 177-191.

Roth, A., Mercier, A., Lepers, C., Hoy, D., Duituturaga, S., Benyon, E., Guillaumot, L. and Souares, Y. (2014). Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an

unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. *Euro Surveillance* **19**(41).

Rudd, P. A., Wilson, J., Gardner, J., Larcher, T., Babarit, C., Le, T. T., Anraku, I., Kumagai, Y., Loo, Y. M., Gale, M., Jr., Akira, S., Khromykh, A. A. and Suhrbier, A. (2012). Interferon response factors 3 and 7 protect against Chikungunya virus hemorrhagic fever and shock. *Journal of Virology* **86**(18): 9888-9898.

Ryan, J., Dave, K., Emmerich, E., Fernandez, B., Turell, M., Johnson, J., Gottfried, K., Burkhalter, K., Kerst, A., Hunt, A., Wirtz, R. and Nasci, R. (2003). Wicking assays for the rapid detection of West Nile and St. Louis encephalitis viral antigens in mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* **40**(1): 95-99.

Salonen, A., Ahola, T. and Kaariainen, L. (2005). Viral RNA replication in association with cellular membranes. *Current Topics in Microbiology and Immunology* **285**: 139-173.

Sam, I. C., Chua, C. L. and Chan, Y. F. (2011). Chikungunya virus diagnosis in the developing world: a pressing need. *Expert Review of Anti-Infective Therapy* **9**(12): 1089-1091.

Santhosh, S. R., Parida, M. M., Dash, P. K., Pateriya, A., Pattnaik, B., Pradhan, H. K., Tripathi, N. K., Ambuj, S., Gupta, N., Saxena, P. and Lakshmana Rao, P. V. (2007). Development and evaluation of SYBR Green I-based one-step real-time RT-PCR assay for detection and quantification of Chikungunya virus. *Journal of Clinical Virology* **39**(3): 188-193.

Sattabongkot, J., Kiattibut, C., Kumpitak, C., Ponlawat, A., Ryan, J. R., Chan, A. S., Dave, K., Wirtz, R. A. and Coleman, R. E. (2004). Evaluation of the VecTest Malaria Antigen Panel assay for the detection of Plasmodium falciparum and P. vivax circumsporozoite protein in anopheline mosquitoes in Thailand. *Journal of Medical Entomology* **41**(2): 209-214.

Sawyer, L. A. (2000). Antibodies for the prevention and treatment of viral diseases. *Antiviral Research* **47**(2): 57-77.

Schilling, S., Emmerich, P., Gunther, S. and Schmidt-Chanasit, J. (2009). Dengue and Chikungunya virus co-infection in a German traveller. *Journal of Clinical Virology* **45**(2): 163-164.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M. C., Lavenir, R., Pardigon, N., Reynes, J. M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M. P., Brehin, A. C., Cubito, N., Despres, P., Kunst, F., Rey, F. A., Zeller, H. and Brisse, S. (2006). Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Medicine* **3**(7): e263.

Schuurman, J., Perdok, G. J., Lourens, T. E., Parren, P. W., Chapman, M. D. and Aalberse, R. C. (1997). Production of a mouse/human chimeric IgE monoclonal antibody to the house dust mite allergen Der p 2 and its use for the absolute quantification of allergen-specific IgE. *Journal of Allergy and Clinical Immunology* **99**(4): 545-550.

Schwartz, O. and Albert, M. L. (2010). Biology and pathogenesis of chikungunya virus. *Nature Reviews. Microbiology* **8**(7): 491-500.

Searle, B. C. (2010). Scaffold: a bioinformatic tool for validating MS/MS-based proteomic studies. *Proteomics* **10**(6): 1265-1269.

Sergon, K., Yahaya, A. A., Brown, J., Bedja, S. A., Mlindasse, M., Agata, N., Allaranger, Y., Ball, M. D., Powers, A. M., Ofula, V., Onyango, C., Konongoi, L. S., Sang, R., Njenga, M. K. and Breiman, R. F. (2007). Seroprevalence of Chikungunya virus infection on Grande Comore Island, union of the Comoros, 2005. *American Journal of Tropical Medicine and Hygiene* **76**(6): 1189-1193.

Setoh, Y. X., Hobson-Peters, J., Prow, N. A., Young, P. R. and Hall, R. A. (2011). Expression of recombinant West Nile virus prM protein fused to an affinity tag for use as a diagnostic antigen. *Journal of Virological Methods* **175**(1): 20-27.

Shin, G., Yost, S. A., Miller, M. T., Elrod, E. J., Grakoui, A. and Marcotrigiano, J. (2012). Structural and functional insights into alphavirus polyprotein processing and pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **109**(41): 16534-16539.

Shukla, J., Khan, M., Tiwari, M., Sannarangaiah, S., Sharma, S., Rao, P. V. and Parida, M. (2009). Development and evaluation of antigen capture ELISA for early clinical diagnosis of chikungunya. *Diagnostic Microbiology and Infectious Disease* **65**(2): 142-149.

Simizu, B., Yamamoto, K., Hashimoto, K. and Ogata, T. (1984). Structural proteins of Chikungunya virus. *Journal of Virology* **51**(1): 254-258.

Simon, F., Tolou, H. and Jeandel, P. (2006). [The unexpected Chikungunya outbreak]. *Revue de Medecine Interne* **27**(6): 437-441.

Simon, T., Kneusel, R., Haubruck, H., Liedvogel, B. and Diarect, A. G. (2001). New ways for the standardization of autoantibody assays: chimeric monoclonal antibodies. *Scandinavian Journal of Clinical and Laboratory Investigation. Supplement* **235**: 91-97.

Singh, I. R., Suomalainen, M., Varadarajan, S., Garoff, H. and Helenius, A. (1997). Multiple mechanisms for the inhibition of entry and uncoating of superinfecting Semliki Forest virus. *Virology* **231**(1): 59-71.

Singh, S. K. and Unni, S. K. (2011). Chikungunya virus: host pathogen interaction. *Reviews in Medical Virology*.

Snyder, A. J. and Mukhopadhyay, S. (2012). The alphavirus E3 glycoprotein functions in a clade-specific manner. *Journal of Virology* **86**(24): 13609-13620.

Soderlund, H. and Ulmanen, I. (1977). Transient association of Semliki Forest virus capsid protein with ribosomes. *Journal of Virology* **24**(3): 907-909.

Sotelo, E., Llorente, F., Rebollo, B., Camunas, A., Venteo, A., Gallardo, C., Lubisi, A., Rodriguez, M. J., Sanz, A. J., Figuerola, J. and Jimenez-Clavero, M. A. (2011). Development and evaluation of a new epitope-blocking ELISA for universal detection of antibodies to West Nile virus. *Journal of Virological Methods* **174**(1-2): 35-41.

Sourisseau, M., Schilte, C., Casartelli, N., Trouillet, C., Guivel-Benhassine, F., Rudnicka, D., Sol-Foulon, N., Le Roux, K., Prevost, M. C., Fsihi, H., Frenkiel, M. P., Blanchet, F., Afonso, P. V., Ceccaldi, P. E., Ozden, S., Gessain, A., Schuffenecker, I., Verhasselt, B., Zamborlini, A., Saib, A., Rey, F. A., Arenzana-Seisdedos, F., Despres, P., Michault, A., Albert, M. L. and Schwartz, O. (2007). Characterization of reemerging chikungunya virus. *PLoS Pathogens* **3**(6): e89.

Sreejith, R., Rana, J., Dudha, N., Kumar, K., Gabrani, R., Sharma, S. K., Gupta, A., Vrati, S., Chaudhary, V. K. and Gupta, S. (2012). Mapping interactions of Chikungunya virus nonstructural proteins. *Virus Research* **169**(1): 231-236.

Staples, J. E., Breiman, R. F. and Powers, A. M. (2009). Chikungunya fever: an epidemiological review of a re-emerging infectious disease. *Clinical Infectious Diseases* **49**(6): 942-948.

Strauss, E. G., De Groot, R. J., Levinson, R. and Strauss, J. H. (1992). Identification of the active site residues in the nsP2 proteinase of Sindbis virus. *Virology* **191**(2): 932-940.

Strauss, E. G., Stec, D. S., Schmaljohn, A. L. and Strauss, J. H. (1991). Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants. *Journal of Virology* **65**(9): 4654-4664.

Strauss, J. H. and Strauss, E. G. (1994a). The alphaviruses: Gene expression, replication, and evolution. *Microbiological Reviews* **58**(3): 491-562.

Strauss, J. H. and Strauss, E. G. (1994b). The alphaviruses: gene expression, replication, and evolution. *Microbiological Reviews* **58**(3): 491-562.

Strohal, R., Kroemer, G., Wick, G. and Kofler, R. (1987). Complete variable region sequence of a nonfunctionally rearranged kappa light chain transcribed in the nonsecretor P3-X63-Ag8.653 myeloma cell line. *Nucleic Acids Research* **15**(6): 2771.

Studies, J. M. C. f. N.-p. (2011). Chemical and biological weapons: possession and programs past and present, James Martin Center for Non-proliferation Studies [online], http://cns.miis.edu/cbw/possess.htm.

Suhrbier, A., Jaffar-Bandjee, M. C. and Gasque, P. (2012). Arthritogenic alphaviruses--an overview. *Nature Reviews. Rheumatology* **8**(7): 420-429.

Suomalainen, M., Liljestrom, P. and Garoff, H. (1992). Spike protein-nucleocapsid interactions drive the budding of alphaviruses. *Journal of Virology* **66**(8): 4737-4747.

Talarmin, F., Staikowsky, F., Schoenlaub, P., Risbourg, A., Nicolas, X., Zagnoli, A. and Boyer, P. (2007). [Skin and mucosal manifestations of chikungunya virus infection in adults in Reunion Island]. *Medecine Tropicale* **67**(2): 167-173.

Tandale, B. V., Sathe, P. S., Arankalle, V. A., Wadia, R. S., Kulkarni, R., Shah, S. V., Shah, S. K., Sheth, J. K., Sudeep, A. B., Tripathy, A. S. and Mishra, A. C. (2009). Systemic involvements and fatalities during Chikungunya epidemic in India, 2006. *Journal of Clinical Virology* **46**(2): 145-149.

Tesh, R. B. (1982). Arthritides caused by mosquito-borne viruses. *Annual Review of Medicine* **33**: 31-40.

Thomas, S., Rai, J., John, L., Schaefer, S., Putzer, B. M. and Herchenroder, O. (2013). Chikungunya virus capsid protein contains nuclear import and export signals. *Virology Journal* **10**: 269.

Thonnon, J., Spiegel, A., Diallo, M., Diallo, A. and Fontenille, D. (1999). [Chikungunya virus outbreak in Senegal in 1996 and 1997]. *Bulletin de la Societe de Pathologie Exotique* **92**(2): 79-82.

Tiwari, M., Parida, M., Santhosh, S. R., Khan, M., Dash, P. K. and Rao, P. V. (2009). Assessment of immunogenic potential of Vero adapted formalin inactivated vaccine derived from novel ECSA genotype of Chikungunya virus. *Vaccine* **27**(18): 2513-2522.

Tsetsarkin, K. A., Vanlandingham, D. L., McGee, C. E. and Higgs, S. (2007). A single mutation in Chikungunya virus affects vector specificity and epidemic potential. *PLoS Pathogens* **3**(12): e201.

Ulmanen, I. (1978). Assembly of Semliki Forest virus nucleocapsid: detection of a precursor in infected cells. *Journal of General Virology* **41**(2): 353-365.

Urdea, M., Penny, L. A., Olmsted, S. S., Giovanni, M. Y., Kaspar, P., Shepherd, A., Wilson, P., Dahl, C. A., Buchsbaum, S., Moeller, G. and Hay Burgess, D. C. (2006). Requirements for high impact diagnostics in the developing world. *Nature* **444 Suppl 1**: 73-79.

Van Bortel, W., Dorleans, F., Rosine, J., Blateau, A., Rousset, D., Matheus, S., Leparc-Goffart, I., Flusin, O., Prat, C., Cesaire, R., Najioullah, F., Ardillon, V., Balleydier, E., Carvalho, L., Lemaitre, A., Noel, H., Servas, V., Six, C., Zurbaran, M., Leon, L., Guinard, A., van den Kerkhof, J., Henry, M., Fanoy, E., Braks, M., Reimerink, J., Swaan, C., Georges, R., Brooks, L., Freedman, J., Sudre, B. and Zeller, H. (2014). Chikungunya outbreak in the Caribbean region, December 2013 to March 2014, and the significance for Europe. *Euro Surveillance* **19**(13).

Vazeille, M., Mousson, L., Martin, E. and Failloux, A. B. (2010). Orally co-Infected Aedes albopictus from La Reunion Island, Indian Ocean, can deliver both dengue and chikungunya infectious viral particles in their saliva. *PLoS Neglected Tropical Diseases* **4**(6): e706.

Vazeille, M., Moutailler, S., Coudrier, D., Rousseaux, C., Khun, H., Huerre, M., Thiria, J., Dehecq, J. S., Fontenille, D., Schuffenecker, I., Despres, P. and Failloux, A. B. (2007). Two Chikungunya Isolates from the Outbreak of La Reunion (Indian Ocean) Exhibit Different Patterns of Infection in the Mosquito, Aedes albopictus. *PLoS ONE* **2**(11): e1168.

Ventoso, I., Sanz, M. A., Molina, S., Berlanga, J. J., Carrasco, L. and Esteban, M. (2006). Translational resistance of late alphavirus mRNA to eIF2alpha phosphorylation: a strategy to overcome the antiviral effect of protein kinase PKR. *Genes and Development* **20**(1): 87-100.

Viennet, E., Knope, K., Faddy, H. M., Williams, C. R. and Harley, D. (2013). Assessing the threat of chikungunya virus emergence in Australia. *Commun Dis Intell Q Rep* **37**(2): E136-143.

Volk, S. M., Chen, R., Tsetsarkin, K. A., Adams, A. P., Garcia, T. I., Sall, A. A., Nasar, F., Schuh, A. J., Holmes, E. C., Higgs, S., Maharaj, P. D., Brault, A. C. and Weaver, S. C. (2010). Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. *Journal of Virology* **84**(13): 6497-6504.

Vrati, S., Fernon, C. A., Dalgarno, L. and Weir, R. C. (1988). Location of a major antigenic site involved in Ross River virus neutralization. *Virology* **162**(2): 346-353.

Wang, D., Suhrbier, A., Penn-Nicholson, A., Woraratanadharm, J., Gardner, J., Luo, M., Le, T. T., Anraku, I., Sakalian, M., Einfeld, D. and Dong, J. Y. (2011). A complex adenovirus vaccine against chikungunya virus provides complete protection against viraemia and arthritis. *Vaccine* **29**(15): 2803-2809.

Wanja, E., Parker, Z. F., Odusami, O., Rowland, T., Dave, K., Dave, S. and Turell, M. J. (2014). Immuno-chromatographic wicking assay for the rapid detection of dengue viral antigens in mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* **51**(1): 220-225.

Warter, L., Lee, C. Y., Thiagarajan, R., Grandadam, M., Lebecque, S., Lin, R. T., Bertin-Maghit, S., Ng, L. F., Abastado, J. P., Despres, P., Wang, C. I. and Nardin, A. (2011). Chikungunya virus envelope-specific human monoclonal antibodies with broad neutralization potency. *Journal of Immunology* **186**(5): 3258-3264.

Watson, R. (2007). Europe witnesses first local transmission of Chikungunya fever in Italy. *BMJ: British Medical Journal* **335**(7619): 532-533.

Weaver, S. C., Hagenbaugh, A., Bellew, L. A., Netesov, S. V., Volchkov, V. E., Chang, G. J., Clarke, D. K., Gousset, L., Scott, T. W., Trent, D. W. and et al. (1993). A comparison of the nucleotide sequences of Eastern and Western Equine Encephalomyelitis viruses with those of other alphaviruses and related RNA viruses. *Virology* **197**(1): 375-390.

Weaver, S. C., Kang, W., Shirako, Y., Rumenapf, T., Strauss, E. G. and Strauss, J. H. (1997). Recombinational history and molecular evolution of western equine encephalomyelitis complex alphaviruses. *Journal of Virology* **71**(1): 613-623.

Weiner, L. M. (2007). Building better magic bullets--improving unconjugated monoclonal antibody therapy for cancer. *Nature Reviews. Cancer* **7**(9): 701-706.

Wong, H. C., Sternini, C., Lloyd, K., De Giorgio, R. and Walsh, J. H. (1996). Monoclonal antibody to VIP: production, characterization, immunoneutralizing activity, and usefulness in cytochemical staining. *Hybridoma* **15**(2): 133-139.

Yap, G., Pok, K. Y., Lai, Y. L., Hapuarachchi, H. C., Chow, A., Leo, Y. S., Tan, L. K. and Ng, L. C. (2010). Evaluation of Chikungunya diagnostic assays: differences in sensitivity of serology assays in two independent outbreaks. *PLoS Neglected Tropical Diseases* **4**(7): e753.

Yathi, K. K., Bhasker, S. and Chinnamma, M. (2013). Determination of B cell epitopes and evaluation of antigen capture ELISA for the earlier diagnosis of CHIK virus using anti-rCHIK E1 rabbit antibodies. *Journal of Immunological Methods* **393**(1-2): 45-52.

Zhang, C. (2012). Hybridoma technology for the generation of monoclonal antibodies. *Methods in Molecular Biology* **901**: 117-135.

Zhao, H., Lindqvist, B., Garoff, H., von Bonsdorff, C. H. and Liljestrom, P. (1994). A tyrosine-based motif in the cytoplasmic domain of the alphavirus envelope protein is essential for budding. *EMBO Journal* **13**(18): 4204-4211.

APPENDIX 1

Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Pyke, A. T., Suhrbier, A. and Hall, R. A. (2013). Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model. *Clin Immunol* **149**(3): 487-497.

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Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model

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Abstract Chikungunya virus (CHIKV) recently caused the largest epidemic ever recorded for this virus involving an estimated 1.4–6.5 million cases, with imported cased reported in over 40 countries. The number of monoclonal antibodies specific for this re-emerging alphavirus is currently limited. Herein we describe the generation and characterisation of five monoclonal antibodies specific for the E2 glycoprotein of CHIKV. The antibodies detected a range of CHIKV isolates in several assays including ELISA, Western blot, immunofluorescence assay (IFA) and immunohistochemistry (IHC) without evidence of cross-reactivity with other alphaviruses. Four antibodies also neutralised CHIKV *in vitro*, two of which provided complete protection against arthritis in a CHIKV mouse model when administered prior to infection. Given the current shortage of widely available reagents for CHIKV, these specific antibodies will be useful not only in research, but may also provide the basis for new diagnostics and treatments.

1. Introduction

Chikungunya virus (CHIKV) belongs to a group of mosquito-transmitted arthritogenic alphaviruses that include the Australian Ross River virus (RRV) and Barmah Forest virus (BFV), the African o'nyong-nyong virus, the South American Mayaro virus, and the globally distributed Sindbis viruses [1]. Alphaviruses are enveloped, single-stranded positive-sense RNA viruses whose ≈11.5 kb genomes encode four non-structural proteins (nsP 1–4) and five structural proteins; capsid, E3, E2, 6K and E1 [2]. CHIKV was first isolated in 1952 in Tanzania and has caused sporadic epidemics of primarily rheumatic disease every 2–50 years mainly in Africa and Asia

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[1]. Since 2004, CHIKV has been responsible for a series of unprecedented outbreaks with an estimated 1.4–6.5 million human infections, with imported cases reported in over 40 countries [1]. Although typically transmitted by *Aedes aegypti* mosquito vectors in urbanised areas, the recent epidemic was associated with the emergence of a new clade of chikungunya viruses, characterised by an amino acid substitution in the CHIKV E1 glycoprotein (A226V), that allowed efficient transmission by *Aedes albopictus* mosquitoes [3]. In the past 30 years, *A. albopictus* has experienced a dramatic global expansion in its geographic distribution [4], and its presence in southern Europe allowed the first autochthonous (endogenously transmitted) CHIKV infections in Italy in 2007 (>200 cases) and in France in 2010 [1].

CHIKV disease in humans is characterised by acute and chronic polyarthralgia/polyarthritis, which usually resolves within weeks to months, but can be protracted [1,5]. The acute phase of disease is often also associated with an abrupt onset of fever, myalgia, and a rash (usually maculopapular) [1,6–9]. Although CHIKV disease is usually self-limiting, the recent epidemic has been associated with some severe disease manifestations and mortality, primarily amongst elderly patients with co-morbidities and the very young [10–13]. During the recent epidemic, mother-to-child transmission was also observed and about half the neonates born to viraemic mothers became infected. About half the infected neonates developed serious forms of CHIKV disease characterised by haemorrhage, disseminated intravascular coagulation, and/or cardiac and neurological manifestations; the latter often leading to permanent disabilities ([1] and refs therein).

Serodiagnosis by IgM and IgG ELISA tests are used as standards for laboratory-based diagnoses of arthritogenic alphavirus diseases, and in-house CHIKV ELISAs have been developed in a number of countries [1]. Detection of the mosquito-borne virus has also been achieved using PCR-based methods; however, blood samples must be taken during the viraemic period [14–16]. Treatment of CHIKV rheumatic disease usually involves the use of analgesics and/or non steroidal anti-inflammatory drugs, with relief often inadequate. There is currently no licensed human vaccine available for any alphavirus, although CHIKV vaccines are in development (e.g. Bharat Biotech) [17]. Neutralizing antibodies are believed to be crucial for providing protection, with polyvalent CHIKV-specific antibodies able to prevent CHIKV infection and disease in mouse models [18,19].

CHIKV is a biosafety level 3 pathogen in most countries and is listed as a US National Institute of Allergy and Infectious Diseases category C priority pathogen [20]. The US Army has long recognised CHIKV as a potential biological weapon, and CHIKV is considered a possible agent for bioterrorism [21]. Centers for Disease Control and Prevention (CDC), and the Pan-American Health Organisation recently completed a preparedness plan for the spread of CHIKV to the Americas due to the fact that all the conditions and vectors exist for such an event [22].

Due to the re-emergence of CHIKV and the potential threat to human health there has been a quest for new research reagents, and improved diagnostics and treatments. Herein we report the generation and characterisation of a panel of monoclonal antibodies (mAbs) to the E2 glycoprotein of CHIKV. These antibodies recognised multiple isolates of CHIKV and did not cross-react with other arthritogenic alphaviruses. We illustrate the use of these monoclonal antibodies in Western blot, IFA, IHC, capture ELISA diagnostic assays and as potential prophylactic biological drugs in a mouse model of CHIKV viraemia and arthritic disease.

2. Material and methods

2.1. Cell and virus culture

Mosquito cells (C6/36—*A. albopictus*) were propagated in RPMI 1640 supplemented with 2% foetal bovine serum. Cultures were passaged by dissociating the cell monolayer from the flask with trypsin/PBS and were incubated at 28 °C. Vero and COS-7L (African green monkey kidney) cell lines were cultured in DMEM and RPMI 1640, respectively, supplemented with 2% foetal bovine serum. Mammalian cells were passaged by dissociating the surface monolayer from the flask with trypsin/EDTA and were cultured at 37 °C with 5% CO_2. Hybridoma cells were expanded in Hybridoma SFM (Gibco, Life Technologies) with 20% foetal bovine serum at 37 °C with 5% CO_2. All cell cultures were supplemented with 50 U penicillin mL^{-1}, 50 μg streptomycin mL^{-1} and 2 mM L-Glutamine (Gibco, Life Technologies).

Viruses used to infect C6/36 cells for fixed-cell ELISA (Clark et al. [23]) included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID: EU404186); CHIKV Asian strain ($CHIKV_{ASIAN}$) (GenBank ID: FJ457921); CHIKV Asian, East Timor strain ($CHIKV_{ET}$) (provided by Dr. Alyssa Pyke, Queensland Health Forensic and Scientific Services); RRV T48 strain (RRV_{T48}) (GenBank ID: GQ433359); Semliki Forest virus (SFV) (GenBank ID: NC_003215); Sindbis virus MRE16 strain ($SINV_{MRE16}$) (GenBank ID: AF492770) and BFV BH2193 strain (BFV_{BH2193}) (GenBank ID: U73745). Briefly, cells seeded in 96-well plates were infected at an M.O.I. of 0.1 and incubated for 3–4 days before the culture supernatant was removed and cells were fixed overnight in 20% acetone, 0.2% bovine serum albumin in phosphate-buffered saline (PBS) at 4 °C. Plates were then air-dried and stored at −20 °C until use.

Mock and virus-infected C6/36 cell monolayers were incubated in a similar manner before cells were rinsed in PBS and disrupted by sonication in the presence of BS9 lysis buffer (120 mM NaCl, 50 mM H_3BO_3, 1% Triton X-100 and 0.1% SDS, pH 9.0). The lysate was clarified by centrifugation at 12,000 ×*g* for 10 min at 4 °C and stored at −20 °C [23].

2.2. Mouse immunisation and hybridoma production

Groups of three to five female BALB/c mice 6–8 weeks of age were immunised subcutaneously (s.c.) onto the ventral side of the ear using a dry-coated nanopatch as described previously [24]. Briefly, mice were given 0.5 μg or 5.0 μg of sucrose gradient-purified γ-irradiated $CHIKV_{MAU}$ antigen (strain 06113879—2006 Mauritius, provided by Dr. Julian Druce, VIDRL, Australia) with Quil-A saponin (Brenntag Biosector) as adjuvant. An identical immunisation was performed three weeks later with a final boost of 5.0 μg $CHIKV_{MAU}$ antigen given four weeks after the second application. Mice were then challenged with live virus as described previously [19,24]. Finally, 20 months later, mice were boosted with 10 μg

of binary-ethyleneimine inactivated $CHIKV_{ASIAN}$ [19] via subcutaneous injection at the base of the tail four days prior to hybridoma production.

2.3. Hybridoma production

Mouse spleens were harvested for hybridoma production by fusion of spleen B cells with MRX63 myeloma cells as previously described [23]. Hybridomas secreting CHIKV-reactive mAbs were identified by fixed-cell ELISA as described below (Section 2.4). Isotype determination of selected monoclonal antibodies was performed using the Mouse Typer isotyping kit (Bio-Rad) according to the manufacturer's instructions.

2.4. Fixed-cell ELISA

Fixed-cell ELISAs were performed as described previously [23,25]. Briefly, acetone-fixed plates of infected C6/36 cells were blocked with TENTC blocking buffer (0.05 M Tris–HCl pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.05% (v/v) Tween 20, 0.2% (w/v) casein) for 1 h at 28 °C prior to probing of fixed antigen with hybridoma culture fluid at starting dilution of 1/10, and then serially diluted 2-fold across the plate. After incubation for 1 h at 28 °C, wells were washed four times with PBS with 0.1% Tween-20 (PBS/T) wash buffer and bound antibodies were detected with a HRP-conjugated goat anti-mouse IgG (DAKO) diluted 1:4000 in blocking buffer. Following a 1 h incubation, the plates were washed six times prior to the addition of 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate solution (0.02% (w/v) ABTS, 0.06% (w/v) H_2O_2 in 200 mM Na_2HPO_4 and 100 mM citric acid solution) for 30 min at 28 °C in the dark. Absorbance was measured at 405 nm using a Labsystems Multiscan EX Type 355 UV plate reader (Pathtec). The criterion for specific recognition of antigen was defined as an OD_{405mn} value of at least 0.25 and at least 2-fold greater than that generated by probing uninfected C6/36 cells with the corresponding antibody dilutions. Control monoclonal antibodies G8, 2F2 and 9E8 specific for RRV, SINV and BFV, respectively, were used in the comparative ELISAs described below [26,27].

2.5. Recombinant CHIKV glycoprotein expression

CHIKV E2 and E1 constructs were generated by amplifying the respective glycoprotein genes, from cDNA synthesised by reverse-transcription PCR of genomic RNA of $CHIKV_{MAU}$, with primer sets CHIKV E3_E2 Forward 5′-ATATAATAGCTAG CATGAGTCTTGCCATCCCAGTTATG-3′, CHIKV E3_E2 Reverse 5′-TTATAATAGGATCCTGTTCTGATGCAGCATA-3′, CHIKV 6K_E1 Forward 5′-AATTAATTGCTAGCATGGCCACATACCAAGAGG-3′, CHIKV 6K_E1 Reverse 5′-ATATATATGGATCCGTGCCTGCTGAAC GACACG-3′, followed by ligation into a pcDNA3.1 (+) vector (Invitrogen) modified to express V5 and histidine tags at the C-terminus of the recombinant proteins. COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were harvested 24 h post-transfection by addition of BS9 lysis buffer and clarified by centrifugation [28]. PNGase F enzyme (Sigma-Aldrich) was used, according to manufacturer's instructions, to cleave N-linked oligosaccharide chains for verification of glycosylation [29].

2.6. Immunofluorescence assay (IFA)

Transfected COS-7L cells were fixed onto glass coverslips with 100% ice-cold acetone and incubated with selected mAbs in hybridoma culture fluid for 1 h at 37 °C. In the case of live virus infection, Vero cells were allowed to grow overnight on glass coverslips before being infected with CHIKV at an M.O.I. of 0.1 for 1 h. Cells were then washed twice with PBS and incubated at 37 °C in complete growth medium. At 72 h post-infection, Vero cells were fixed and incubated with anti-CHIKV mAbs at a 1/20 dilution as described above. Coverslips were then washed and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) diluted 1:500 in blocking buffer for 1 h at 37 °C, followed by Hoechst 33342 stain (Invitrogen; 1:1000 in PBS) for 5 min. Coverslips were mounted with ProLong Gold Anti-Fade reagent (Invitrogen) and imaged using a Zeiss LSM 510 META confocal microscope.

2.7. Western blot

CHIKV antigens were prepared as crude, CHIKV-infected cell lysate or transfected COS-7L cell lysates as previously described [29]. For reduction and carboxymethylation of antigens, lysates were reconstituted in 0.1 M Tris–HCl and reduced with 1 M dithiothreitol (DTT). Samples were then gently treated with streaming nitrogen for 30 s before being heated at 95 °C for 5 min. Reduced lysates were cooled at 4 °C for 5 min prior to the addition of iodoacetic acid to a final concentration of 1 M. Samples were gassed as described above and incubated at 37 °C in the dark for 1 h [23]. All CHIKV antigens were prepared in 4× NuPAGE LDS sample buffer (Invitrogen) and heated at 95 °C for 5 min. The proteins were resolved on 4–12% Bis–Tris precast SDS-PAGE gels (Invitrogen), transferred onto Hybond C nitrocellulose membranes (Amersham) and immune-stained as previously described [23]. Briefly, membranes were blocked with TENTC blocking buffer for 1 h at room temperature prior to the addition of CHIKV-specific mAbs or anti-CHIKV polyclonal mouse sera diluted 1/20, unless otherwise stated, in blocking buffer. After incubation for another hour, membranes were washed thrice with PBS/T wash buffer and bound antibodies were detected with a HRP-conjugated goat anti-mouse IgG diluted 1:4000 in blocking buffer. The blots were incubated for a further 1 h before being washed three times with PBS/T wash buffer. Finally, blots were developed in DAB substrate solution (1.5 mM 3,3′-diaminobenzidine, 0.06% (v/v) H_2O_2 in PBS, pH 7.2) for 15 min before reactions were terminated by rinsing with PBS or ddH_2O.

2.8. Competitive binding assays

Competitive binding between CHIKV E2 mAbs was assessed as described previously [30]. Briefly, purified mAbs were biotinylated using the BiotinTag kit (Sigma), according to the manufacturer's instructions. The competitive binding ELISAs were performed in 96 well plates coated with a lysate of $CHIKV_{MAU}$-infected C6/36 cells diluted 1/500 in coating buffer (0.05 M sodium carbonate/bicarbonate, pH 9.6). After washing, a pre-defined optimal saturating concentration of each of the unlabelled mAbs (1.3A2–1.25 μg/mL; 4.6 F5–0.078 μg/mL; 4.10C12–1.25 μg/mL; 5.2B2–20 μg/mL; 5.2H8–0.156 μg/mL; and 4G2–0.4 μg/mL) was added for 1 h at 28 °C. Without

Table 1 Reactivity of CHIKV E2-specific mAbs towards various CHIKV strains and other alphaviruses in ELISA.

Monoclonal antibody	Reactivity in fixed-cell ELISA						
	CHIKV (Mau)	CHIKV (Asian)	CHIKV (ET)	RRV (T48)	SFV	SINV (MRE16)	BFV (BH2193)
1.3 A2 $_{IgG2a}$	+ + + + +	+ + + +	+ + + + +	–	–	–	–
4.6F5 $_{IgG2a}$	+ + + +	+ + + + +	+ + + + +	–	–	–	–
4.10 C12 $_{IgG2a}$	+ + + + +	+ + + + +	+ + + + +	–	–	–	–
5.2 B2 $_{IgG2a}$	+ + + +	+ + + + +	+ + + + +	–	–	–	–
5.2H8 $_{IgG2a}$	+ + + +	+ + + + +	+ + + + +	–	–	–	–
G8 $_{IgG2a}$	+	+	+	+ + + + +	+ + + + +	–	+ + +
2F2 $_{IgG1}$	–	–	–	–	–	+ + + +	–
9E8	–	–	N.D.	N.D.	–	–	+ + + + +
2B2 $_{IgG2a}$	–	–	–	–	–	–	–

The dilution producing the maximum mean absorbance reading was scored as: +++++, OD > 1.0; ++++, OD = 0.75 to 1.0; +++, OD = 0.5 to 0.75; ++, OD = 0.3 to 0.5; +, OD = 0.25 to 0.3.

washing, a pre-defined optimal non-saturating dilution of each biotin-labelled 'competitor' mAb (1.3A2–1.25 μg/mL; 4.6 F5–1.25 μg/mL; 4.10C12–2.5 μg/mL; 5.2B2–20 μg/mL; and 5.2H8–5 μg/mL) was added for 1 h at 28 °C. After washing six times with PBS/T, horseradish peroxidase (HRP)-conjugated streptavidin (Invitrogen) was added and incubated for 30 min. The wells were washed prior to incubation with ABTS substrate solution.

2.9. Antigen-capture ELISA

U-bottom PVC 96-well plates (BD Falcon) were coated with 1 μg of purified anti-E2 mAb at 4 °C overnight in coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6). Purified soluble E2 (sE2) antigen, kindly provided by Stefan Metz (Laboratory of Virology, Wageningen University, The Netherlands), was captured by incubation for 1 h at 28 °C, prior to another hour of blocking as described previously. A second biotinylated anti-E2 mAb that binds a different epitope from the capturing mAb was used as a detecting antibody. After incubation for 1 h at 28 °C, enzyme activity was visualised by the addition of ABTS substrate solution as described previously [23]. Concentrations of purified sE2 protein were initially determined using a BCA Protein Assay kit (Pierce) by the provider (Stefan Metz, personal communications) and confirmed in our laboratory using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific).

2.10. Microneutralisation assay

Virus neutralisation tests were performed on Vero cells as described previously [31] with minor modifications. Antibodies tested were subjected to heat inactivation at 56 °C for 30 min before being diluted with DMEM supplemented with 2% FBS. Polyclonal mouse serum was used at an initial dilution of 1:20, while purified CHIKV-specific mAbs were used at a starting concentration of 5 μg per well. Each well was then examined microscopically for cytopathic effect (CPE) and fixed with 4% paraformaldehyde in PBS for 1 h at room temperature before staining with 0.05% crystal violet for validation of cell viability. Neutralisation titres were expressed as the reciprocal of the highest serum dilution where CPE did not occur.

2.11. Passive immunisation with mAb and virus challenge

Six-week old C57BL/6 mice were subcutaneously inoculated with 400 μg (~15 mg/kg) of monoclonal antibody in 100 μL

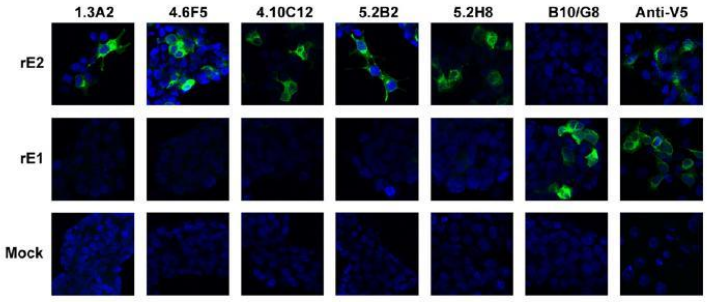

Figure 1 Monoclonal antibody reactivity by IFA with acetone-fixed monolayers of COS-7L cells transfected with rE2 and rE1 constructs. Cells were probed with respective mAbs before incubation with an anti-mouse Alexa Fluro 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining. B10/G8 mAbs were generated to the E1 protein of RRV and are cross-reactive with the E1 protein of CHIKV. Successful expression of rE2 and rE1 was demonstrated using anti-V5 mAb.

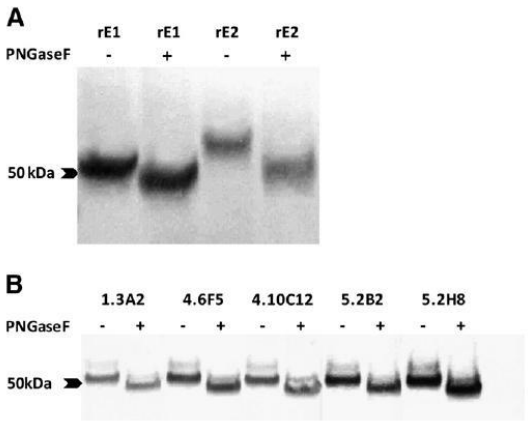

Figure 2 Western blot analyses of full-length recombinant E1 and E2 proteins expressed in COS-7L cells with or without PNGaseF treatment. (A) Boiled, unreduced lysates of transfected COS-7L cells expressing rE1 and rE2 were digested with PNGase F (+) or undigested (−); recombinant CHIKV proteins detected using anti-V5 mAb. (B) All five anti-E2 mAbs recognised both glycosylated and unglycosylated forms of E2 under unreduced conditions.

PBS one day prior to challenge with CHIKV (LR2006-OPY1), and viraemia and foot swelling (arthritis) monitored as described previously [19]. Viral titres were calculated as $\log_{10}$ 50% cell culture infectious dose per mL of serum ($\log_{10}$ $CCID_{50}$/ml) using 10-fold serial dilutions on C6/36 cells and detection of infection in individual wells using Vero cell cytopathic effects as described [19]. The detection cut off is 2 $\log_{10}$ $CCID_{50}$. Foot swelling over time was determined blinded and as a group average of the percentage increase in foot height × width (in the metatarsal region) for each foot compared with the same foot on day 0 [32].

2.12. Immunohistochemical staining of CHIKV-infected mouse tissue

Tissue samples from control and CHIKV-infected mouse foot pads were fixed in 10% neutral buffered formalin for 24 h at room temperature and decalcified with 15% EDTA in 0.1% phosphate buffer over 10 days before being embedded in paraffin wax. Tissue sections, 5 μm thick, from uninfected and $CHIKV_{REUNION}$-infected mice were collected onto charged slides and deparaffinised with three changes of xylene (2 min each), followed by rehydration through a series of graded ethanol concentrations and finally water. Sections were subjected to antigen retrieval by heating at 95 °C in a citrate-buffer, pH 6 (Target Retrieval Solution, DAKO) for 25 min followed by a 20 min cooling period at room temperature, or by incubation with Proteinase K (DAKO) at room temperature for 10 min. A series of blocking steps were performed at room temperature incubation with (i) Peroxidase Block (DAKO) for 10 min, (ii) 0.15 M glycine in PBS for 15 min, (iii) Antibody Diluent with Background-Reducing Components (DAKO) for 30 min, with a brief rinse in Tris-buffered saline with 0.1% Tween-20 (TBS/T) between each step. The tissue samples were then incubated in 1 μg/mL of purified 4.10C12 mAb at 4 °C overnight, prior to 15 min washing with TBS/T. Antibody binding was visualised using the anti-mouse IgG Envision kit (DAKO) according to the manufacturer's instruction. Sections were counterstained with Meyer's hematoxylin, mounted with Glycergel Mounting Medium (DAKO) and examined under a Nikon Eclipse 51E microscope. Digital micro-photographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and processed with the NIS-Elements F software.

2.13. Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 19; SPSS, Chicago, IL, USA). For comparison of two samples, the *t*-test was used when the difference in the variances was less than four and skewness was greater than minus two and kurtosis was less than two. Otherwise, non-parametric tests were used; the Mann–Whitney *U* test if the difference in the variances was less than four and the Kolmogorov–Smirnov test if the difference in the variances was greater than 4.

3. Results

3.1. Production of CHIKV-specific monoclonal antibodies

Five hybridomas secreting antibodies reactive to CHIKV proteins were cloned by limit dilution and mAbs were harvested as clarified culture supernatant. The reactivity of these mAbs (1.3A2, 4.6F5, 4.10C12, 5.2B2 and 5.2H8) towards CHIKV and related alphaviruses were determined by fixed-cell ELISA (Table 1). While all five mAbs recognised $CHIKV_{MAU}$, $CHIKV_{ASIAN}$ and $CHIKV_{ET}$ strains by ELISA, none were able to detect proteins from fixed cells infected with the related viruses RRV, SFV, SINV or BFV, suggesting that all

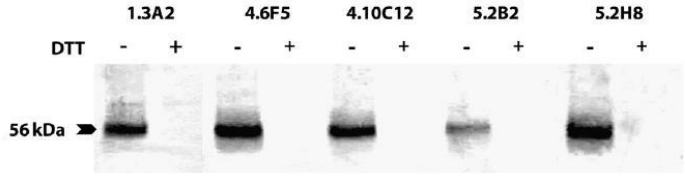

Figure 3 Reaction of anti-E2 mAbs to DTT-reduced (+) and unreduced (−) CHIKV-infected cell lysates. DTT breaks disulphide bonds in proteins while the carboxymethylation process caps the free cysteine residues, preventing the reformation of disulphide bonds, and thus, proteins from refolding. All proteins were separated on 4–12% Bis–Tris SDS-PAGE gels.

mAbs produced were specific to CHIKV. All five mAbs were of the IgG_{2A} isotype.

3.2. CHIKV-specific mAbs recognise epitopes on the E2 glycoprotein

To determine the protein specificity of these mAbs, recombinant full-length CHIKV E1 (rE1) and E2 (rE2) glycoproteins were expressed in COS-7L cells. Successful expression of the glycoproteins, along with their C-terminus V5-His epitope tag, was confirmed with the use of anti-V5 mAbs (Fig. 1). CHIKV mAbs produced in this study reacted with rE2 in IFA, while rE1 was detected using E1-specific cross-reactive RRV mAbs (B10 and G8) produced previously [33]. Alphavirus E1 and E2 proteins have asparagine (N) linked glycosylation sites; however, the number of glycosylation sites vary between different species of viruses [34–36]. CHIKV-E1 is predicted to be glycosylated at N141, while CHIKV-E2 was expected to have two glycosylation sites at N263 and N273 [37]. Undigested rE1 and rE2 were shown to be ~51 kDa and ~56 kDa, respectively, while their PNGase F-digested counterparts were ~48 kDa and ~50 kDa in size (Fig. 2A). This difference in mass is consistent with the single N-linked glycosylation site in E1 and two sites within E2 as elaborated in a previous study [38]. Further analysis also revealed that the binding of all five anti-E2 mAbs was not dependent on the glycosylation status of the protein as they were capable of detecting the E2 protein in both its original and deglycosylated forms (Fig. 2B).

3.3. Anti-E2 mAbs recognise reduction-sensitive conformational epitopes

Crude lysates from $CHIKV_{ASIAN}$ infected cells were either reduced with DTT and free sulfhydryl groups carboxymethylated to prevent reformation of disulphide bonds, or were left unreduced prior to SDS-PAGE. Subsequent immunoblotting illustrated that mAbs 1.3A2, 4.6F5, 4.10C12, 5.2B2 and 5.2H8 all recognised rE2 in unreduced samples, but not in DTT-reduced/carboxymethylated samples (Fig. 3). These results show that the E2-specific mAbs recognised epitopes dependent on intact cysteine residues, suggesting a requirement for secondary structure provided by disulphide bonds for antibody binding.

3.4. Competitive binding assay reveals mAbs bind to three different epitopes of E2

To determine the topology of the epitopes recognised by the mAbs on the E2 protein, each antibody was tested in competitive binding assays using ELISA. These assays showed that the five E2-specific mAbs collectively recognise at least three different epitopes on the protein (Fig. 4). Monoclonal antibodies 1.3A2, 4.6F5 and 4.10C12 inhibited each another, suggesting the epitopes they recognise overlap. Meanwhile, mAbs 5.2B2 and 5.2H8 did not compete with each other, or with any of the other three mAbs, indicating that they bind to different epitopes within E2.

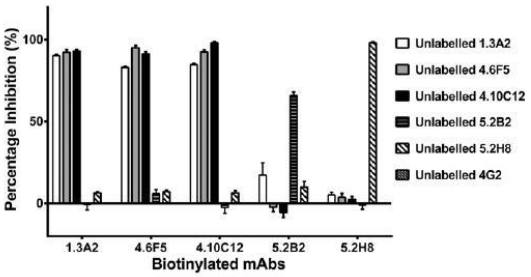

Figure 4 Competitive-binding profiles of E2-specific mAbs in ELISA. Antigens in lysates of $CHIKV_{MAU}$-infected Vero cells were adsorbed to 96-well plates at a 1/500 dilution prior to incubation with a saturating dilution of purified, unlabelled anti-E2 mAbs. Without washing, non-saturating dilutions of biotinylated mAbs were then added as 'competitor' antibodies to respective wells. The mean absorbance reading (OD_{405nm}) of four replicates were plotted with bars showing standard error of mean (SEM). 4G2 is a control mAb specific to the E protein of flaviviruses. Assay was optimised to obtain complete inhibition of each biotinylated mAb by its homologous unlabelled competitor.

3.5. Use of E2-specific mAbs in antigen-capture assay allows for sensitive detection of CHIKV E2 protein

A double-antibody sandwich antigen-capture ELISA was performed to determine the ability of the anti-E2 mAbs to detect viral proteins in samples. After a series of empirical trials to determine the optimal antibody pairing, two mAbs that bound to different locations of the E2 protein were selected for this assay—purified, unlabelled mAb 1.3A2 was utilised to capture the target antigen, sE2 protein, while a second biotinylated anti-E2 mAb, 5.2H8, was applied as the

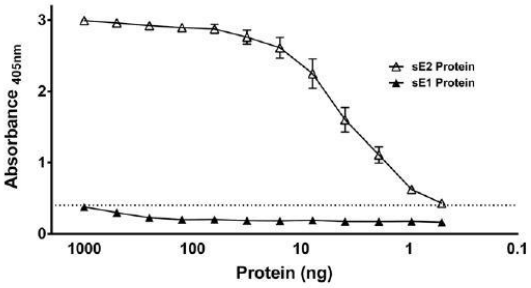

Figure 5 Detection of purified soluble E2 protein by anti-E2 mAb in an antigen-capture ELISA. Unlabelled mAb 1.3A2 was coated onto 96-well plates at 1 μg/well prior to incubation with target sE2 protein. The detecting antibody, biotinylated 5.2H8 mAb, was used at 400 ng per well. The mean absorbance reading (OD_{405nm}) of eight replicates from two independent experiments were plotted with bars showing SEM. Dotted line represents the cut-off point, for a positive result, derived from the no antigen control plate (mean OD_{405nm} reading × 2), while sE1 protein (n = 2) was utilised as a negative control for the assay.

detecting antibody. Fig. 5 shows that this assay is capable of detecting the sE2 protein in the picogram range.

3.6. Anti-E2 mAbs exhibit virus neutralisation *in vitro*

Virus neutralisation assays were undertaken to determine whether the CHIKV E2-specific mAbs were able to neutralise virus *in vitro*. Micro-neutralisation tests were performed on Vero cells and revealed that mAbs 1.3A2, 4.6F5 and 4.10C12 were able to efficiently neutralise different CHIKV isolates (Table 2), with $CHIKV_{MAU}$ and $CHIKV_{ASIAN}$ representing the East/South African and Asian phylogroups, respectively [39]. Monoclonal antibody 5.2B2 showed some neutralisation of $CHIKV_{ASIAN}$, but not with $CHIKV_{MAU}$, while mAb 5.2H8 failed to neutralise either CHIKV isolate even at the highest antibody concentration.

3.7. MAbs 1.3A2 and 4.6F5 protect against viraemia and arthritis

A recent study described the use of an adult wild-type mouse model of CHIKV infection and disease, which mimics both the viraemia and rheumatic symptoms seen in humans [19]. This mouse model has, for instance, been used to test the efficacy of a number of CHIKV vaccines [24,40]. The model was used to determine whether neutralizing mAbs produced to CHIKV E2 in this study could provide prophylactic protection from viraemia and disease after live CHIKV challenge. Based on their efficient neutralisation of CHIKV strains *in vitro*, and the higher yields of purified monoclonal antibodies from their hybridoma cell lines, mAbs 1.3A2 and 4.6F5 were chosen for the *in vivo* study. Mice were inoculated with 400 μg of purified 1.3A2 or 4.6F5 mAb one day prior to challenge with CHIKV. Mice that received these antibodies showed no signs of arthritis as measured by foot swelling (Fig. 6A) and showed a mean 7-8 log reduction in viraemia when compared with controls (Fig. 6B). In contrast, control mice (that received PBS or a non-alphavirus reactive mAb) developed clear signs of arthritis that peaked days 6–7 post infection (Fig. 6A), demonstrating the expected [19] viraemia peaking at 7–8 $\log_{10}$ $CCID_{50}$ on day 2 (Fig. 6B). These results show that mAbs 1.3A2 and 4.6F5 can provide complete protection against CHIKV arthritis and significantly suppress viraemia when administered prior to infection.

Table 2 Neutralisation activity of E2 mAbs against two CHIKV strains.

	Amount of antibody (μg) required for 80% virus neutralisation[a]	
	CHIKV MAURITIUS	CHIKV ASIAN
Antibody	100 infectious units	100 infectious units
CHIKV 1.3A2	2.5	2.5
CHIKV 4.6F5	2.5	2.5
CHIKV 4.10C12	2.5	2.5
CHIKV 5.2B2	>5	5
CHIKV 5.2H8	>5	>5
Mouse sera ($CHIKV_{ASIAN}$)[b]	1/100	1/100
Isotype control (2B2)[c]	>5	>5

[a] Determined microscopically as 80% reduction in CPE in four replicate wells per antibody dilution.
[b] Dilution factor used instead of antibody concentration.
[c] 2B2 is an IgG2A mAb that reacts to E protein of WNV.

3.8. Detection of CHIKV antigens in tissue of infected mice by IHC

To assess the use of the E2-specific mAbs to detect CHIKV in tissues samples, IHC was performed on formalin-fixed, paraffin-embedded samples previously prepared from foot pads of $IRF3/7^{-/-}$ mice infected with $CHIKV_{REUNION}$ [32]. Although staining of infected cells was observed with the use of all anti-CHIKV E2 mAbs when fixed with acetone, 4.10C12 was the only mAb that gave clear reactivity with formalin-fixed tissue sections (Fig. 7). The detection of CHIKV within keratinocytes in infected mice tissue shown here is consistent with previous studies [32]. These results indicate that the mAbs produced in this study can be applied to IHC to specifically recognise CHIKV antigens in formalin-fixed tissue sections.

4. Discussion

The persistent spread of CHIKV into previously naive areas along with its high epidemic potential poses a serious global threat to areas where competent *Aedes* mosquito vectors exist in abundance. The social and economic impact of CHIKV epidemics underlines the exigency for accurate diagnostics and effective anti-viral therapeutics, since vaccines are not yet available.

This study describes the generation and characterisation of five mAbs specific to the E2 glycoprotein of CHIKV. Their specific recognition of three strains of CHIKV (i.e. $CHIKV_{MAU}$, $CHIKV_{ASIAN}$ and $CHIKV_{ET}$) that represent the major global lineages of the virus [39], along with the lack of cross-reaction with other alphaviruses species examined, indicate these antibodies will be useful as specific research and diagnostic tools.

The alphavirus E2 protein is thought to be involved in virus attachment to host cell receptors and contains critical binding sites for neutralizing antibodies as shown in SINV models [41–43]. This is consistent with *in vitro* virus neutralizing activity of mAbs 1.3A2, 4.6F5 and to a lesser extent 4.10C12. These mAbs potentially bind to either the receptor-binding site of the CHIKV E2 protein or an epitope capable of interfering with virus attachment. When we further assessed the ability of mAbs 1.3A2 and 4.6F5 to provide *in vivo* protection in a previously established arthritis mouse model of CHIKV infection [19], a single dose of either mAb gave complete protection against disease progression in all test animals ($n = 8$). Foot swelling was absent in mice inoculated with the anti-CHIKV mAbs, indicating that the virus had a significant reduction in its ability to proliferate in its primary site of injection. Moreover, the absence of viraemia indicated that the virus introduced was most likely neutralised and was less capable of spreading. The levels of *in vivo* protection provided by mAbs in this study were similar to that recently reported for human anti-CHIKV mAbs by Warter et al. [44,45]. When

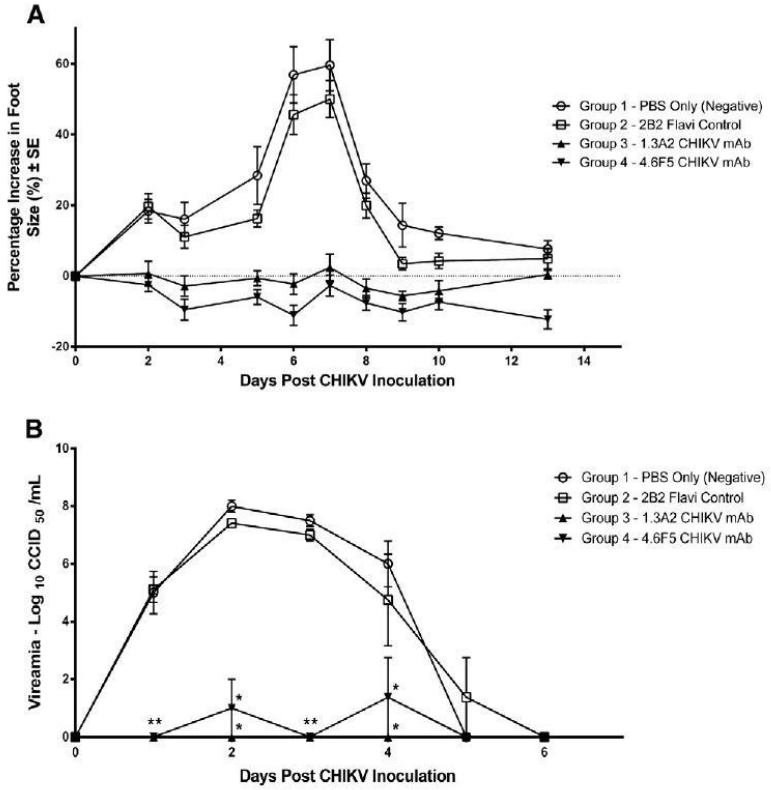

Figure 6 Foot swelling and virus replication in mice inoculated with CHIKV-specific mAbs. (A) Arthritis after CHIKV infection. Mice were given 400 μg of mAbs 1.3A2, 4.6F5, 2B2 (anti-flavivirus isotype control) or PBS subcutaneously, one day prior to infection with CHIKV. The data is presented as a group average of the percentage increase in foot height × width for each foot compared with the same foot on day 0 (n = 8 ft per group). PBS and 2B2 groups were significantly different from 1.3A2 and 4.6F5 groups on days 2–10; $p \leq 0.034$, t-tests and Kolmogorov–Smirnov tests. (B) Serum viraemia. The same mice as in A were assessed for viraemia in serum collected at the indicated times (n = 4 mice per group). * indicates $p \leq 0.037$, Kolmogorov–Smirnov tests.

inoculated into AGR.129 (IFN-$\alpha/\beta/\gamma R^{-/-/-}$ and RAG-2^{-}) mice, they showed that at least 250 μg of mAb was required to achieve 80–100% protection from a lethal dose of CHIKV [45], as compared to complete protection obtained with 400 μg of mAb in the wild-type mouse model of CHIKV disease used in the current study. Although the practicality of utilizing such antibodies for therapeutic purposes is often questioned, neutralizing antibodies might be useful to prevent mother-to-child infections [1]. These results demonstrate the potential of these anti-E2 mAbs to be used prophylactically during an outbreak and warrant future investigation.

Our competitive binding studies indicated that the mAbs produced in this study recognised at least three spatially separated epitopes on E2. These data assisted in the design of a capture assay for CHIKV detection. By selecting two mAbs that bound to different epitopes on the E2 protein, an antigen-capture ELISA was developed for the sensitive detection of CHIKV antigen. Using a purified recombinant CHIKV E2 protein as a standard, this assay was capable of detecting the antigen at picogram levels, demonstrating similar sensitivity to capture assays to detect West Nile virus (WNV) antigen in mosquitoes [46]. Further validation of the sensitivity and specificity of this assay is now required using clinical samples to assess its application as a diagnostic assay for the identification of CHIKV in acute infections. These mAbs could also be incorporated into an antigen-capture dip stick format for the detection of CHIKV in mosquito samples, as previously described for WNV [47]. Our demonstration that CHIKV E2 antigens can also be detected in infected mouse tissue samples by mAb 4.10C12 in IHC identifies a further application for these reagents as specific tools for the study of CHIKV pathogenesis.

5. Conclusions

In summary, we have produced a panel of mAbs capable of distinguishing CHIKV from other common alphaviruses that cause similar disease syndromes in Australia, representing

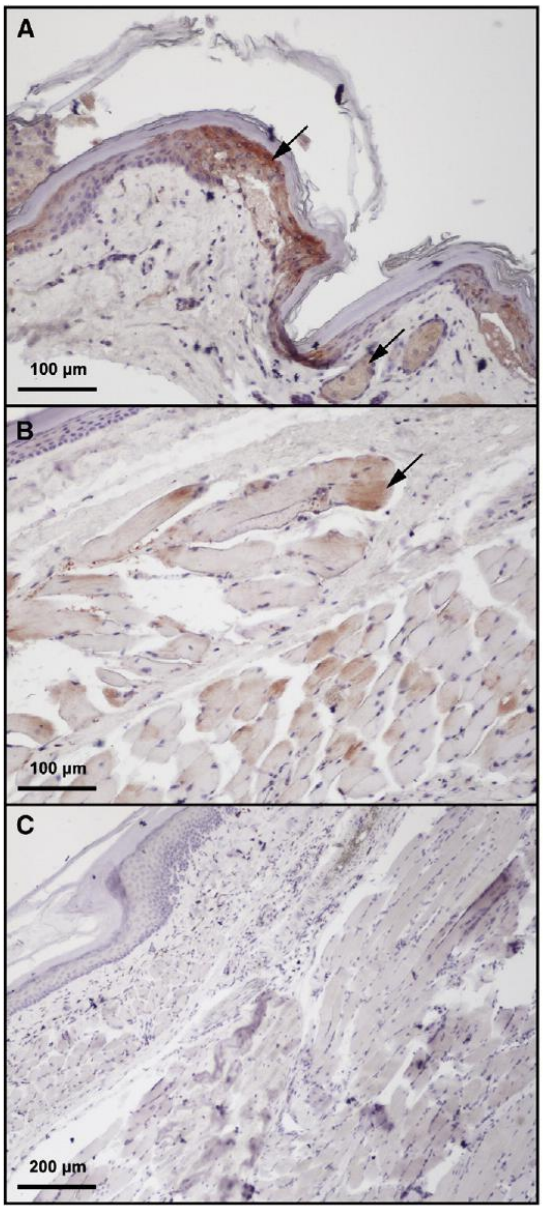

Figure 7 Immunohistochemical staining for CHIKV using E2-specific mAb 4.10C12. Five micron sections of formalin-fixed paraffin-embedded uninfected or CHIKV-infected feet tissue of IRF3/7$^{-/-}$ mice were stained using 4.10C12. One microgram of purified 4.10C12 was incubated with tissue sections overnight at 4 °C. Antibody binding was visualised using the anti-mouse IgG Envision kit, DAKO. Staining was observed in (A) epidermal keratinocytes (top arrow) and sweat glands (bottom arrow), and (B) skeletal striated muscle cells, while no staining was observed in the uninfected control (C); A and B were photographed at a magnification of 20×, while the uninfected control C is shown at 10× zoom.

potentially useful reagents to specifically detect CHIKV in mosquito vectors and/or acute patient samples. However, further studies are required to evaluate their specificity and sensitivity in various diagnostic formats. Successful employment of these antibodies, especially 4.10C12, to detect CHIKV antigen in tissues by IHC further reinforces

their potential in a wide variety of diagnostic assays for clinical or research purposes. The evaluation of these anti-CHIKV mAbs against additional medically significant alphaviruses will also allow them to be utilised as diagnostic tools in other regions of the world. Furthermore, we have demonstrated that these E2-specific mAbs neutralise virus both *in vitro* and *in vivo*, highlighting their potential as prophylactic agents against CHIKV infection when genetically engineered into humanised forms. These mAbs will also be useful for studying the structure and function of E2 in viral assembly and virus–host interactions [48]. Finally, Metz and colleagues have shown that the CHIKV E2 protein can be a good vaccine candidate and the mAbs developed in this study could prove extremely valuable in terms of monitoring the antigenic authenticity and production of such a vaccine for the disease [38].

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2013.10.004.

References

[1] A. Suhrbier, M.C. Jaffar-Bandjee, P. Gasque, Arthritogenic alphaviruses—an overview, Nat. Rev. Rheumatol. 8 (2012) 420–429.
[2] J.H. Strauss, E.G. Strauss, The alphaviruses: gene expression, replication, and evolution, Microbiol. Rev. 58 (1994) 491–562.
[3] N.P. Kumar, R. Joseph, T. Kamaraj, P. Jambulingam, A226V mutation in virus during the 2007 chikungunya outbreak in Kerala, India, J. Gen. Virol. 89 (2008) 1945–1948.
[4] L. Lambrechts, T.W. Scott, D.J. Gubler, Consequences of the expanding global distribution of *Aedes albopictus* for dengue virus transmission, PLoS Negl. Trop. Dis. 4 (2010) e646.
[5] J.J. Hoarau, M.C. Jaffar Bandjee, P. Krejbich Trotot, T. Das, G. Li-Pat-Yuen, B. Dassa, M. Denizot, E. Guichard, A. Ribera, T. Henni, F. Tallet, M.P. Moiton, B.A. Gauzere, S. Bruniquet, Z. Jaffar Bandjee, P. Morbidelli, G. Martigny, M. Jolivet, F. Gay, M. Grandadam, H. Tolou, V. Vieillard, P. Debre, B. Autran, P. Gasque, Persistent chronic inflammation and infection by Chikungunya arthritogenic alphavirus in spite of a robust host immune response, J. Immunol. 184 (2010) 5914–5927.
[6] R.B. Tesh, Arthritides caused by mosquito-borne viruses, Annu. Rev. Med. 33 (1982) 31–40.
[7] M.C. Robinson, An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. I. Clinical features, Trans. R. Soc. Trop. Med. Hyg. 49 (1955) 28–32.
[8] J.E. Staples, R.F. Breiman, A.M. Powers, Chikungunya fever: an epidemiological review of a re-emerging infectious disease, Clin. Infect. Dis. 49 (2009) 942–948.
[9] G. Borgherini, P. Poubeau, F. Staikowsky, M. Lory, N. Le Moullec, J.P. Becquart, C. Wengling, A. Michault, F. Paganin, Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients, Clin. Infect. Dis. 44 (2007) 1401–1407.
[10] M.C. Jaffar-Bandjee, D. Ramful, B.A. Gauzere, J.J. Hoarau, P. Krejbich-Trotot, S. Robin, A. Ribera, J. Selambarom, P. Gasque, Emergence and clinical insights into the pathology of Chikungunya virus infection, Expert Rev. Anti-Infect. Ther. 8 (2010) 987–996.
[11] B.V. Tandale, P.S. Sathe, V.A. Arankalle, R.S. Wadia, R. Kulkarni, S.V. Shah, S.K. Shah, J.K. Sheth, A.B. Sudeep, A.S. Tripathy, A.C. Mishra, Systemic involvements and fatalities during Chikungunya epidemic in India, 2006, J. Clin. Virol. 46 (2009) 145–149.
[12] A. Economopoulou, M. Dominguez, B. Helynck, D. Sissoko, O. Wichmann, P. Quenel, P. Germonneau, I. Quatresous, Atypical Chikungunya virus infections: clinical manifestations, mortality and risk factors for severe disease during the 2005–2006 outbreak on Reunion, Epidemiol. Infect. 137 (2009) 534–541.
[13] D. Mavalankar, P. Shastri, T. Bandyopadhyay, J. Parmar, K.V. Ramani, Increased mortality rate associated with Chikungunya epidemic, Ahmedabad, India, Emerg. Infect. Dis. 14 (2008) 412–415.
[14] C.J. Edwards, S.R. Welch, J. Chamberlain, R. Hewson, H. Tolley, P.A. Cane, G. Lloyd, Molecular diagnosis and analysis of Chikungunya virus, J. Clin. Virol. 39 (2007) 271–275.
[15] P. Laurent, K. Le Roux, P. Grivard, G. Bertil, F. Naze, M. Picard, F. Staikowsky, G. Barau, I. Schuffenecker, A. Michault, Development of a sensitive real-time reverse transcriptase PCR assay with an internal control to detect and quantify chikungunya virus, Clin. Chem. 53 (2007) 1408–1414.
[16] S.R. Santhosh, M.M. Parida, P.K. Dash, A. Pateriya, B. Pattnaik, H.K. Pradhan, N.K. Tripathi, S. Ambuj, N. Gupta, P. Saxena, P.V. Lakshmana Rao, Development and evaluation of SYBR Green I-based one-step real-time RT-PCR assay for detection and quantification of Chikungunya virus, J. Clin. Virol. 39 (2007) 188–193.
[17] W. Akahata, Z.Y. Yang, H. Andersen, S. Sun, H.A. Holdaway, W.P. Kong, M.G. Lewis, S. Higgs, M.G. Rossmann, S. Rao, G.J. Nabel, A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection, Nat. Med. 16 (2010) 334–338.
[18] T. Couderc, N. Khandoudi, M. Grandadam, C. Visse, N. Gangneux, S. Bagot, J.F. Prost, M. Lecuit, Prophylaxis and therapy for Chikungunya virus infection, J. Infect. Dis. 200 (2009) 516–523.
[19] J. Gardner, I. Anraku, T.T. Le, T. Larcher, L. Major, P. Roques, W.A. Schroder, S. Higgs, A. Suhrbier, Chikungunya virus arthritis in adult wild-type mice, J. Virol. 84 (2010) 8021–8032.
[20] National Institute of Allergy and Infectious Diseases, NIAID Category A, B, and C Priority Pathogens, National Institute of Allergy and Infectious Diseases, 2011. ([online], http://www.niaid.nih.gov/topics/biodefenserelated/biodefense/pages/cata.aspx).
[21] James Martin Center for Non-proliferation Studies, Chemical and Biological Weapons: Possession and Programs Past and Present, James Martin Center for Non-proliferation Studies, 2011. ([online], http://cns.miis.edu/cbw/possess.htm).

[22] Pan American Health Organization, Preparedness and Response for Chikungunya Virus: Introduction in the Americas, Pan American Health Organization, Washington, D.C., 2011, ISBN 978-92-75-11632-6.
[23] D.C. Clark, M. Lobigs, E. Lee, M.J. Howard, K. Clark, B.J. Blitvich, R.A. Hall, In situ reactions of monoclonal antibodies with a viable mutant of Murray Valley encephalitis virus reveal an absence of dimeric NS1 protein, J. Gen. Virol. 88 (2007) 1175–1183.
[24] T.W. Prow, X. Chen, N.A. Prow, G.J. Fernando, C.S. Tan, A.P. Raphael, D. Chang, M.P. Ruutu, D.W. Jenkins, A. Pyke, M.L. Crichton, K. Raphaelli, L.Y. Goh, I.H. Frazer, M.S. Roberts, J. Gardner, A.A. Khromykh, A. Suhrbier, R.A. Hall, M.A. Kendall, Nanopatch-targeted skin vaccination against West Nile Virus and Chikungunya virus in mice, Small 6 (2010) 1776–1784.
[25] R.A. Hall, G.W. Burgess, B.H. Kay, Type-specific monoclonal antibodies produced to proteins of Murray Valley encephalitis virus, Immunol. Cell Biol. 66 (Pt 1) (1988) 51–56.
[26] P. Clancy, Production and Application of Monoclonal Antibodies to the Australian Alphaviruses, MSc Thesis School of Tropical Veterinary Science and Agriculture, James Cook University of North Queensland, Townsville, Queensland, Australia, 1991.
[27] N.M. Oliveira, A.K. Broom, J.S. Mackenzie, D.W. Smith, M.D. Lindsay, B.H. Kay, R.A. Hall, Epitope-blocking enzyme-linked immunosorbent assay for detection of antibodies to Ross River virus in vertebrate sera, Clin. Vaccine Immunol. 13 (2006) 814–817.
[28] J. Hobson-Peters, P. Toye, M.D. Sanchez, K.N. Bossart, L.F. Wang, D.C. Clark, W.Y. Cheah, R.A. Hall, A glycosylated peptide in the West Nile virus envelope protein is immunogenic during equine infection, J. Gen. Virol. 89 (2008) 3063–3072.
[29] Y.X. Setoh, J. Hobson-Peters, N.A. Prow, P.R. Young, R.A. Hall, Expression of recombinant West Nile virus prM protein fused to an affinity tag for use as a diagnostic antigen, J. Virol. Methods 175 (2011) 20–27.
[30] R.A. Hall, S.E. Tan, B. Selisko, R. Slade, J. Hobson-Peters, B. Canard, M. Hughes, J.Y. Leung, E. Balmori-Melian, S. Hall-Mendelin, K.B. Pham, D.C. Clark, N.A. Prow, A.A. Khromykh, Monoclonal antibodies to the West Nile virus NS5 protein map to linear and conformational epitopes in the methyltransferase and polymerase domains, J. Gen. Virol. 90 (2009) 2912–2922.
[31] R.A. Hall, A.K. Broom, A.C. Hartnett, M.J. Howard, J.S. Mackenzie, Immunodominant epitopes on the NS1 protein of MVE and KUN viruses serve as targets for a blocking ELISA to detect virus-specific antibodies in sentinel animal serum, J. Virol. Methods 51 (1995) 201–210.
[32] P.A. Rudd, J. Wilson, J. Gardner, T. Larcher, C. Babarit, T.T. Le, I. Anraku, Y. Kumagai, Y.M. Loo, M. Gale Jr., S. Akira, A.A. Khromykh, A. Suhrbier, Interferon response factors 3 and 7 protect against Chikungunya virus hemorrhagic fever and shock, J. Virol. 86 (2012) 9888–9898.
[33] A.K. Broom, R.A. Hall, C.A. Johansen, N. Oliveira, M.A. Howard, M.D. Lindsay, B.H. Kay, J.S. Mackenzie, Identification of Australian arboviruses in inoculated cell cultures using monoclonal antibodies in ELISA, Pathology 30 (1998) 286–288.
[34] D. Burke, K. Keegstra, Carbohydrate structure of Sindbis virus glycoprotein E2 from virus grown in hamster and chicken cells, J. Virol. 29 (1979) 546–554.
[35] C.M. Rice, J.H. Strauss, Nucleotide sequence of the 26S mRNA of Sindbis virus and deduced sequence of the encoded virus structural proteins, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 2062–2066.
[36] R.L. Knight, K.L. Schultz, R.J. Kent, M. Venkatesan, D.E. Griffin, Role of N-linked glycosylation for sindbis virus infection and replication in vertebrate and invertebrate systems, J. Virol. 83 (2009) 5640–5647.
[37] N. Blom, T. Sicheritz-Ponten, R. Gupta, S. Gammeltoft, S. Brunak, Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence, Proteomics 4 (2004) 1633–1649.
[38] S.W. Metz, C. Geertsema, B.E. Martina, P. Andrade, J.G. Heldens, M.M. van Oers, R.W. Goldbach, J.M. Vlak, G.P. Pijlman, Functional processing and secretion of Chikungunya virus E1 and E2 glycoproteins in insect cells, Virol. J. 8 (2011) 353.
[39] I. Schuffenecker, I. Iteman, A. Michault, S. Murri, L. Frangeul, M.C. Vaney, R. Lavenir, N. Pardigon, J.M. Reynes, F. Pettinelli, L. Biscornet, L. Diancourt, S. Michel, S. Duquerroy, G. Guigon, M.P. Frenkiel, A.C. Brehin, N. Cubito, P. Despres, F. Kunst, F.A. Rey, H. Zeller, S. Brisse, Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak, PLoS Med. 3 (2006) e263.
[40] D. Wang, A. Suhrbier, A. Penn-Nicholson, J. Woraratanadharm, J. Gardner, M. Luo, T.T. Le, I. Anraku, M. Sakalian, D. Einfeld, J.Y. Dong, A complex adenovirus vaccine against chikungunya virus provides complete protection against viraemia and arthritis, Vaccine 29 (2011) 2803–2809.
[41] M. Kielian, C. Chanel-Vos, M. Liao, Alphavirus entry and membrane fusion, Viruses 2 (2010) 796–825.
[42] N.L. Davis, D.F. Pence, W.J. Meyer, A.L. Schmaljohn, R.E. Johnston, Alternative forms of a strain-specific neutralizing antigenic site on the Sindbis virus E2 glycoprotein, Virology 161 (1987) 101–108.
[43] E.G. Strauss, D.S. Stec, A.L. Schmaljohn, J.H. Strauss, Identification of antigenically important domains in the glycoproteins of Sindbis virus by analysis of antibody escape variants, J. Virol. 65 (1991) 4654–4664.
[44] L. Warter, C.Y. Lee, R. Thiagarajan, M. Grandadam, S. Lebecque, R.T. Lin, S. Bertin-Maghit, L.F. Ng, J.P. Abastado, P. Despres, C.I. Wang, A. Nardin, Chikungunya virus envelope-specific human monoclonal antibodies with broad neutralization potency, J. Immunol. 186 (2011) 3258–3264.
[45] J. Fric, S. Bertin-Maghit, C.I. Wang, A. Nardin, L. Warter, Treatment of Chikungunya virus infection using human monoclonal antibodies, J. Infect. Dis. 207 (2) (2012) 319–322.
[46] A.R. Hunt, R.A. Hall, A.J. Kerst, R.S. Nasci, H.M. Savage, N.A. Panella, K.L. Gottfried, K.L. Burkhalter, J.T. Roehrig, Detection of West Nile virus antigen in mosquitoes and avian tissues by a monoclonal antibody-based capture enzyme immunoassay, J. Clin. Microbiol. 40 (2002) 2023–2030.
[47] R.S. Nasci, K.L. Gottfried, K.L. Burkhalter, J.R. Ryan, E. Emmerich, K. Dave, Sensitivity of the VecTest antigen assay for eastern equine encephalitis and western equine encephalitis viruses, J. Am. Mosq. Control Assoc. 19 (2003) 440–444.
[48] W. Akahata, G.J. Nabel, A specific domain of the chikungunya virus e2 protein regulates particle formation in human cells: implications for alphavirus vaccine design, J. Virol. 86 (2012) 8879–8883.

APPENDIX 2

Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Suhrbier, A. and Hall, R. A. (2014). Monoclonal antibodies specific for the capsid protein of chikungunya virus suitable for multiple applications. *J Gen Virol* **in press**. as doi:10.1099/jgv.0.000002.

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Short Communication

Monoclonal antibodies specific for the capsid protein of chikungunya virus suitable for multiple applications

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Chikungunya virus (CHIKV) is a mosquito-borne pathogen responsible for epidemics of debilitating arthritic disease. The recent outbreak (2004–2014) resulted in an estimated 1.4–6.5 million cases, with imported cases reported in nearly 40 countries. The development of CHIKV-specific diagnostics and research tools is thus highly desirable. Herein we describe the generation and characterization of the first mAbs specific for the capsid protein (CP) of CHIKV. The antibodies recognized isolates representing the major genotypes of CHIKV, as well as several other alphaviruses, and were reactive in a range of assays including ELISA, Western blot, immunofluorescence and immunohistochemistry (IHC). We have also used the anti-CP mAb 5.5G9 in IHC studies to show that capsid antigen is persistently expressed 30 days post-infection in cells with macrophage morphology in a mouse model of chronic CHIKV disease. These antibodies may thus represent useful tools for further research, including investigations into the structure and function of CHIKV CP, and as valuable reagents for CHIKV detection in a range of settings.

Chikungunya virus (CHIKV) is the aetiological agent of CHIK fever, first described in 1952 during an epidemic in Tanzania, East Africa (Lumsden, 1955; Robinson, 1955). CHIKV belongs to the *Alphavirus* genus within the *Togaviridae* family and is an enveloped, single-stranded positive-sense RNA virus (Strauss & Strauss, 1994). The 11.5 kb alphavirus genome is capped at its 5′ end and polyadenylated at its 3′ end, and encodes four non-structural proteins (nsP1 to nsP4) and five structural proteins (capsid, E3, E2, 6K and E1) (Strauss & Strauss, 1994).

CHIKV is transmitted to humans by *Aedes aegypti,* and recently also *Aedes albopictus*, mosquitoes. Acute CHIKV disease is characterized by a rapid onset of fever, myalgia and often a rash (usually maculopapular), with chronic disease characterized by episodic, often debilitating, polyarthralgia/polyarthritis (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Suhrbier *et al.*, 2012). The largest epidemic of CHIKV disease ever reported began in 2004 and has since been responsible for up to 6.5 million human cases, primarily in Africa and Asia, with imported cases reported in over 40 countries (Munasinghe *et al.*, 1966; Lam *et al.*, 2001; Renault *et al.*, 2007; Rezza *et al.*, 2007; Grandadam *et al.*, 2011; Suhrbier *et al.*, 2012; Horwood *et al.*, 2013; Van Bortel *et al.*, 2014). The continued activity of the initial epidemic in conjunction with additional emerging events has led to independent outbreaks in other parts of the globe, such as in Australasia and the Caribbean (Horwood *et al.*, 2013; Viennet *et al.*, 2013; Van Bortel *et al.*, 2014). During the recent epidemic, CHIKV was also clearly associated with occasional severe disease manifestations and mortality, the latter primarily amongst elderly patients with co-morbidities and the very young (Mavalankar *et al.*, 2008; Economopoulou *et al.*, 2009; Tandale *et al.*, 2009; Jaffar-Bandjee *et al.*, 2010).

The alphavirus capsid protein (CP) is a multifunctional protein that has been shown to act as a serine protease for self-cleavage, bind viral genomic RNA and other CP molecules during nucleocapsid formation, and interact with viral spike proteins during virion formation and egress (Choi *et al.*, 1991). The CP of CHIKV consists of 261 amino acids, which form two major domains. The N-terminal domain has a high degree of positive charge

†These authors contributed equally to this work.

A supplementary figure is available with the online Supplementary Material.

implicated in non-specific RNA binding, while the C-terminal domain harbours the globular protease and the binding site for the spike protein (Hong *et al.*, 2006).

The re-emergence of CHIKV, attributable in large part to a mutation allowing efficient transmission by *A. albopictus*, and the current risk it poses to human health, has prompted the demand for new diagnostic and research reagents. Herein we report the generation and characterization of the first monoclonal antibodies (mAbs) to the CP of CHIKV and describe their use in a variety of assays.

CHIKV isolates obtained for these studies included CHIKV Mauritius strain ($CHIKV_{MAU}$) (GenBank ID EU404186); CHIKV Asian, Thailand strain ($CHIKV_{THAI}$) (GenBank ID FJ457921) and CHIKV Asian, East Timor strain ($CHIKV_{ET}$). To generate mAbs against CHIKV viral proteins, BALB/c mice 6–8 weeks of age were immunized with purified inactivated antigen ($CHIKV_{MAU}$), challenged with live virus ($CHIKV_{THAI}$), followed by a final boost 20 months later with inactivated antigen ($CHIKV_{THAI}$) 4 days prior to fusions for hybridoma production as described previously (Goh *et al.*, 2013). Hybridomas were screened for production of CHIKV-reactive antibodies using fixed-cell ELISA, and positive hybridoma cultures were cloned twice by limit dilution as previously described (Hall *et al.*, 1988; Clark *et al.*, 2007). Eleven hybridomas secreting antibodies reactive to CHIKV proteins were expanded in Hybridoma SFM (Gibco Life Technologies) with 20 % FBS at 37 °C with 5 % CO_2, before being weaned off all FBS for the harvesting and clarification of mAbs as culture fluid. Reactivity of these mAbs (1.7B2, 4.1H11, 4.8E2, 4.10A11, 5.1B12, 5.2F8, 5.2H7, 5.4G8, 5.5A11, 5.5D11 and 5.5G9) to various CHIKV strains and related alphaviruses were determined by fixed-cell ELISA (Table 1).

Each mAb recognized the three CHIKV strains used in this study ($CHIKV_{MAU}$, $CHIKV_{THAI}$ and $CHIKV_{ET}$) with similar intensity in ELISA, suggesting the epitopes are highly conserved amongst these strains. However, the varying degree of reactivity between the mAbs to the CHIKV antigens, as measured by OD_{405}, is likely due to their recognition of different binding sites on the CP and variation in the binding affinity between individual mAbs. To further assess their reactivity towards other closely related alphaviruses, each mAb was also tested against Ross River virus (RRV) T48 strain (GenBank ID GQ433359); Semliki Forest virus (SFV) (GenBank ID NC_003215) and Sindbis virus (SINV) MRE16 strain (GenBank ID AF492770). Three of the mAbs, 5.2H7, 5.5D11 and 5.5G9, reacted with antigens of SFV, RRV and SINV in ELISA, while the remaining eight mAbs recognized SFV and/or RRV but not SINV (Table 1). The isotype of each mAb was also determined using the Mouse typer isotyping kit (Bio-Rad) according to the manufacturer's instructions and found to be either IgG_1 or IgG_{2A} (Table 1). When tested for viral neutralizing activity in a micro-neutralization assay (Goh *et al.*, 2013), none of the mAbs neutralized any of the three CHIKV strains *in vitro* (data not shown).

To determine their viral protein specificity, each mAb was assessed for specificity against CHIKV antigens in infected Vero cell lysate by Western blot (Goh *et al.*, 2013). $CHIKV_{MAU}$ antigens were prepared in 4× NuPAGE LDS sample buffer (Invitrogen) and heated at 95 °C for 5 min. For reduced antigens, 10 mM DTT was added prior to

Table 1. Reactivity of CHIKV CP-specific mAbs towards various CHIKV strains and other alphaviruses in ELISA

Monoclonal antibody*	Reactivity in fixed-plate ELISA					
	$CHIKV_{MAU}$	$CHIKV_{THAI}$	$CHIKV_{ET}$	RRV_{T48}	SFV	$SINV_{MRE16}$
$5.2H7_{IgG1}$	++++	++++	+++++	+++++	+++++	++++
$5.5D11_{IgG1}$	++++	++++	++++	+++++	+++++	++++
$5.5G9_{IgG2A}$	+++++	+++++	+++++	+++++	+++++	+++++
$1.7B2_{IgG1}$	+++++	+++++	+++++	++	−	−
$4.1H1_{IgG1}$	+++++	+++++	+++++	++	+	−
$5.1B12_{IgG2A}$	+++++	+++++	+++++	+++	+++	−
$5.5A11_{IgG1}$	+++++	++++	+++++	++	+++++	−
$4.8E2_{IgG2A}$	+++	+++	+++	++	+++	−
$4.10A11_{IgG1}$	+++	+++	+++	++	+++	−
$5.2F8_{IgG2A}$	+++	++	+++	++	++	−
$5.4G8_{IgG1}$	+++	++	++	++	++	−
$G8_{IgG2A}$†	+	+	+	+++++	+++++	−
$2F2_{IgG1}$‡	−	−	−	−	−	++++

*The optimal mAb dilution producing the maximum mean OD_{405} reading on $CHIKV_{MAU}$ antigen was determined empirically for each mAb and used for assessment for other virus strains. Scoring: +++++, OD>1.0; ++++, OD=0.75–1.0; +++, OD=0.5–0.75; ++, OD=0.3–0.5; +, OD=0.25–0.3.

†mAb G8 was generated to the E1 protein of RRV and is cross-reactive with CHIKV and SFV (Oliveira *et al.*, 2006).

‡mAb 2F2 was previously raised to the Australian prototype strain MRM39, and has been shown to be SINV-specific.

heating. The proteins were resolved on 4–12 % Bistris precast SDS-PAGE gels (Invitrogen), transferred onto Hybond C nitrocellulose membranes (GE Healthcare), immune-stained and developed as previously described (Clark *et al.*, 2007). To assess the glycosylation status of the target antigen, lysates were treated with PNGase F (Sigma-Aldrich) according to the manufacturer's instructions, prior to analysis by Western blot. All antibodies reacted to a protein band of ~36 kDa, in samples that were reduced or unreduced, as well as PNGase F-treated and untreated material, consistent with recognition of the unglycosylated CHIKV CP (Fig. 1a).

Specificity towards the CHIKV CP was confirmed by testing the mAbs for reactivity with recombinant $CHIKV_{MAU}$ CP (rCap) expressed in COS-7L cells by means of immunofluorescence assay (IFA). $CHIKV_{MAU}$ CP constructs were generated by amplifying the respective CP genes from cDNA synthesized by reverse-transcription PCR of genomic RNA of $CHIKV_{MAU}$, with primer set 'CHIKV Capsid Forward pcDNA' 5′-TATATA GCTAGC ATG GAGTTCATCCCA-ACCCAA-3′, 'CHIKV Capsid Reverse pcDNA' 5′-TATATA GGATCC ACTCCACTCTTCGGCCCC-3′, followed by ligation into a pcDNA3.1 (+) vector (Invitrogen) modified to express V5 and histidine tags at the C terminus of the recombinant proteins. COS-7L cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Expression of recombinant E1 (rE1) and E2 (rE2) of $CHIKV_{MAU}$ was also carried out for

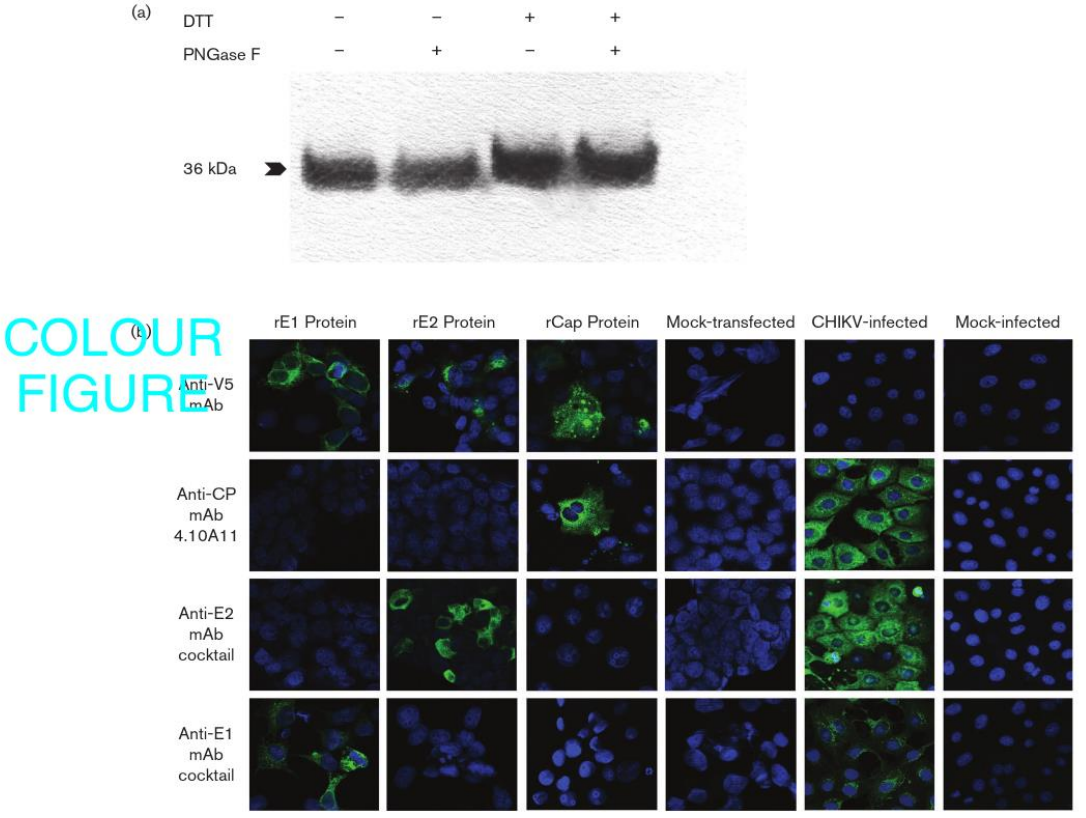

Fig. 1. mAb reactivity in Western blot and IFA. (a) Representative mAb 4.10A11 reactions against boiled, reduced (DTT^+) or unreduced (DTT^-) lysates of $CHIKV_{MAU}$-infected C6/36 cells treated with (+) or without (–) PNGase F. (b) IFA staining of 4.10A11 against transfected/$CHIKV_{MAU}$-infected cells. Cells were probed with ~~the respective mAbs~~ before incubation with an anti-mouse Alexa Fluor 488 conjugate (green) and Hoechst 33342 (blue) for nuclear staining. A cocktail of five mAbs generated in a previous study was used for the detection of CHIKV E2 (Goh *et al.*, 2013). B10/G8 mAbs (anti-E1 cocktail) were generated to the E1 protein of RRV and are cross-reactive with the E1 protein of CHIKV (Oliveira *et al.*, 2006). Successful expression of recombinant proteins was demonstrated using anti-V5 mAb.

2

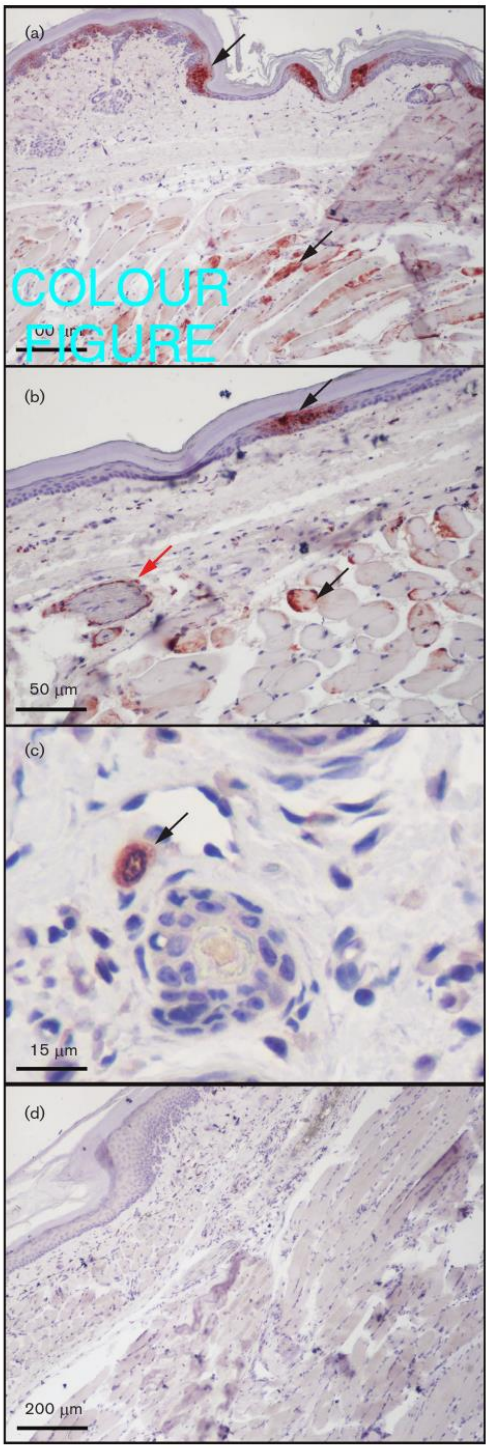

Fig. 2. IHC-labelling for CHIKV antigen using capsid-specific mAb 5.5G9. Labelling was observed in epidermal keratinocytes (a, b, top black arrow), skeletal striated muscle cells (a, b, bottom black arrow) and perineural cells (b, red arrow) in tissue sections of acutely infected IRF3/7$^{-/-}$ mice. Macrophage-like cells within connective tissue of CHIKV-infected WT mouse feet 30 days post-infection also stained positive with 5.5G9 (c). No reactivity was observed in the uninfected control (d). Digital micrographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and the NIS-Elements F software and are reproduced without further manipulation. 3

reference controls as previously described (Goh *et al.*, 2013). Transfected COS-7L cells were fixed onto glass coverslips with 100 % ice-cold acetone and incubated with selected mAbs in hybridoma culture fluid for 1 h at 37 °C. In the case of live virus infection, Vero cells were allowed to grow overnight on glass coverslips before being infected with CHIKV at an m.o.i. of 0.1 for 1 h. Cell monolayers were then washed twice with PBS and incubated at 37 °C in complete growth medium. At 24 h post-infection, Vero cells were fixed and incubated with anti-CHIKV mAbs as described above. Coverslips were then stained, mounted and imaged as described by Goh *et al.* (2013). All 11 mAbs reacted with cells expressing rCap or cells infected with CHIKV, but not the mock-infected/transfected cells or those expressing CHIKV rE1 or rE2 (Figs 1b and S1, available in the online Supplementary Material).

To assess the use of the CHIKV CP-specific mAbs to detect CHIKV in tissues samples, immunohistochemistry (IHC) was performed, as previously described in detail (Goh *et al.*, 2013), on formalin-fixed, paraffin-embedded samples previously prepared from feet of IRF3/7$^{-/-}$ mice infected with CHIKV$_{\text{REUNION}}$ (Rudd *et al.*, 2012) and from wild-type (WT) mice 30 days post-infection. Briefly, deparaffinized sections were subjected to antigen retrieval by heating at 95 °C in a citrate buffer, pH 6 (Target Retrieval Solution, DAKO) for 25 min followed by a 20 min cooling period at room temperature. Following a series of blocking steps, the sections were incubated with undiluted hybridoma culture supernatant of mAb 5.5G9 at 4 °C overnight. Preliminary studies showed that this mAb gave the most intense signal in IHC (data not shown). Antibody binding was visualized using the anti-mouse IgG Envision kit (Dako). Sections were counterstained with Meyer's haematoxylin, mounted and examined under a Nikon Eclipse 51E microscope. Digital micro-photographs were captured using a Nikon DS-Fi1 camera with a DS-U2 unit and processed with the NIS-Elements F software. Clear staining of keratinocytes and skeletal muscle cells was observed in samples from acutely infected IRF3/7$^{-/-}$ mice with the use of mAb 5.5G9 (Fig. 2), consistent with previous *in situ* hybridization studies in these interferon α/β-response-deficient mice (Rudd *et al.*, 2012).

An ongoing issue for the field of alphaviral arthritis is understanding the aetiology of chronic inflammatory

disease. Persistence of CHIKV RNA and protein was reported in occasional macrophages (i) in a chronic CHIKV patient 18 months post-onset of disease in the face of a robust host immune response (Hoarau *et al.*, 2010), and (ii) in cynomolgus macaques (*Macaca fascicularis*) 44 days post-CHIKV infection by *in situ* hybridization (Labadie *et al.*, 2010), but has never been described in a mouse model, possibly due to the lack of sensitive reagents. Using the 5.5G9 mAb, we were able to detect CP antigen in scattered macrophage-like cells in connective tissue of the inoculated foot from WT mice 30 days post-infection (Fig. 2). This previously reported mouse model of acute infection and disease (Gardner *et al.*, 2010) thus recapitulates a key feature of chronic disease seen in humans. CP expression on day 30 – well beyond the 4–6 day viraemic period – in this model further supports the view that CHIKV antigen expression persists long-term and is the likely cause of chronic inflammatory disease (Robinson, 1955; Tesh, 1982; Borgherini *et al.*, 2007; Staples *et al.*, 2009; Labadie *et al.*, 2010; Suhrbier *et al.*, 2012). In addition, the ability specifically to detect CHIKV CP-positive cells in paraformaldehyde-fixed, paraffin wax-embedded and decalcified tissue further illustrates the utility of 5.5G9 for CHIKV research. The 5.5G9 mAb may also prove particularly useful for studies of viral persistence, as it allowed the immune-labelling of rare cells with even low levels of CHIKV CP antigen in fixed tissue sections. CHIKV is a biosafety level 3 organism (thus tissue samples must be fixed prior to removal from the biosafety level 3 facility), with joints – necessitating decalcification – and associated tissues often the focus of research for this arthritogenic alphavirus.

In this paper, we report, we believe, the first mAbs generated to the CHIKV CP, and demonstrate their reactivity in ELISA, Western blot and IFA. Our findings suggest these mAbs represent useful research tools and have strong potential in a wide variety of applications. In addition, we have shown that CHIKV antigen can be detected in infected mouse tissue samples by mAb 5.5G9 in IHC. This identifies a further application for these reagents as specific tools for the study of CHIKV pathogenesis. The mAbs generated in this study also recognized different strains of CHIKV ($CHIKV_{MAU}$, $CHIKV_{THAI}$ and $CHIKV_{ET}$) representing the two major global lineages of the virus (Asian and East/Central/South African) (Schuffenecker *et al.*, 2006). Furthermore, the three mAbs (5.2H7, 5.5D11 and 5.5G9) that also reacted strongly with the non-CHIKV alphaviruses tested here will also be useful research tools for studying CP in related alphaviruses. The mAbs described in this paper are available from the authors upon request.

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References

Borgherini, G., Poubeau, P., Staikowsky, F., Lory, M., Le Moullec, N., Becquart, J. P., Wengling, C., Michault, A. & Paganin, F. (2007). Outbreak of chikungunya on Reunion Island: early clinical and laboratory features in 157 adult patients. *Clin Infect Dis* **44**, 1401–1407.

Choi, H. K., Tong, L., Minor, W., Dumas, P., Boege, U., Rossmann, M. G. & Wengler, G. (1991). Structure of Sindbis virus core protein reveals a chymotrypsin-like serine proteinase and the organization of the virion. *Nature* **354**, 37–43.

Clark, D. C., Lobigs, M., Lee, E., Howard, M. J., Clark, K., Blitvich, B. J. & Hall, R. A. (2007). *In situ* reactions of monoclonal antibodies with a viable mutant of Murray Valley encephalitis virus reveal an absence of dimeric NS1 protein. *J Gen Virol* **88**, 1175–1183.

Economopoulou, A., Dominguez, M., Helynck, B., Sissoko, D., Wichmann, O., Quenel, P., Germonneau, P. & Quatresous, I. (2009). Atypical chikungunya virus infections: clinical manifestations, mortality and risk factors for severe disease during the 2005–2006 outbreak on Réunion. *Epidemiol Infect* **137**, 534–541.

Goh, L. Y., Hobson-Peters, J., Prow, N. A., Gardner, J., Bielefeldt-Ohmann, H., Pyke, A. T., Suhrbier, A. & Hall, R. A. (2013). Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model. *Clin Immunol* **149**, 487–497.

Grandadam, M., Caro, V., Plumet, S., Thiberge, J. M., Souarès, Y., Failloux, A. B., Tolou, H. J., Budelot, M., Cosserat, D. & other authors (2011). Chikungunya virus, southeastern France. *Emerg Infect Dis* **17**, 910–913.

Hall, R. A., Burgess, G. W. & Kay, B. H. (1988). Type-specific monoclonal antibodies produced to proteins of Murray Valley encephalitis virus. *Immunol Cell Biol* **66**, 51–56.

Hoarau, J. J., Jaffar Bandjee, M. C., Krejbich Trotot, P., Das, T., Li-Pat-Yuen, G., Dassa, B., Denizot, M., Guichard, E., Ribera, A. & other authors (2010). Persistent chronic inflammation and infection by chikungunya arthritogenic alphavirus in spite of a robust host immune response. *J Immunol* **184**, 5914–5927.

Hong, E. M., Perera, R. & Kuhn, R. J. (2006). Alphavirus capsid protein helix I controls a checkpoint in nucleocapsid core assembly. *J Virol* **80**, 8848–8855.

Horwood, P., Bande, G., Dagina, R., Guillaumot, L., Aaskov, J. & Pavlin, B. (2013). The threat of chikungunya in Oceania. *Western Pacific Surveill Response J* **4**, 8–10.

Jaffar-Bandjee, M. C., Ramful, D., Gauzere, B. A., Hoarau, J. J., Krejbich-Trotot, P., Robin, S., Ribera, A., Selambarom, J. & Gasque, P. (2010). Emergence and clinical insights into the pathology of chikungunya virus infection. *Expert Rev Anti Infect Ther* **8**, 987–996.

Labadie, K., Larcher, T., Joubert, C., Mannioui, A., Delache, B., Brochard, P., Guigand, L., Dubreil, L., Lebon, P. & other authors (2010). Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. *J Clin Invest* **120**, 894–906.

Lam, S. K., Chua, K. B., Hooi, P. S., Rahimah, M. A., Kumari, S., Tharmaratnam, M., Chuah, S. K., Smith, D. W. & Sampson, I. A.

(2001). Chikungunya infection – an emerging disease in Malaysia. *Southeast Asian J Trop Med Public Health* **32**, 447–451.

Lumsden, W. H. (1955). An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952–53. II. General description and epidemiology. *Trans R Soc Trop Med Hyg* **49**, 33–57.

Mavalankar, D., Shastri, P., Bandyopadhyay, T., Parmar, J. & Ramani, K. V. (2008). Increased mortality rate associated with chikungunya epidemic, Ahmedabad, India. *Emerg Infect Dis* **14**, 412–415.

Munasinghe, D. R., Amarasekera, P. J. & Fernando, C. F. (1966). An epidemic of dengue-like fever in Ceylon (chikungunya – a clinical and haematological study. *Ceylon Med J* **11**, 129–142.

Oliveira, N. M., Broom, A. K., Mackenzie, J. S., Smith, D. W., Lindsay, M. D., Kay, B. H. & Hall, R. A. (2006). Epitope-blocking enzyme-linked immunosorbent assay for detection of antibodies to Ross River virus in vertebrate sera. *Clin Vaccine Immunol* **13**, 814–817.

Renault, P., Solet, J. L., Sissoko, D., Balleydier, E., Larrieu, S., Filleul, L., Lassalle, C., Thiria, J., Rachou, E. & other authors (2007). A major epidemic of chikungunya virus infection on Réunion Island, France, 2005–2006. *Am J Trop Med Hyg* **77**, 727–731.

Rezza, G., Nicoletti, L., Angelini, R., Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S. & other authors (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* **370**, 1840–1846.

Robinson, M. C. (1955). An epidemic of virus disease in Southern Province, Tanganyika territory, in 1952–1953. I. Clinical features. *Trans R Soc Trop Med Hyg* **49**, 28–32.

Rudd, P. A., Wilson, J., Gardner, J., Larcher, T., Babarit, C., Le, T. T., Anraku, I., Kumagai, Y., Loo, Y. M. & other authors (2012). Interferon response factors 3 and 7 protect against chikungunya virus hemorrhagic fever and shock. *J Virol* **86**, 9888–9898.

Schuffenecker, I., Iteman, I., Michault, A., Murri, S., Frangeul, L., Vaney, M. C., Lavenir, R., Pardigon, N., Reynes, J. M. & other authors (2006). Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. *PLoS Med* **3**, e263.

Staples, J. E., Breiman, R. F. & Powers, A. M. (2009). Chikungunya fever: an epidemiological review of a re-emerging infectious disease. *Clin Infect Dis* **49**, 942–948.

Strauss, J. H. & Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**, 491–562.

Suhrbier, A., Jaffar-Bandjee, M. C. & Gasque, P. (2012). Arthritogenic alphaviruses – an overview. *Nature Rev Rheumatol* **8**, 420–429.

Tandale, B. V., Sathe, P. S., Arankalle, V. A., Wadia, R. S., Kulkarni, R., Shah, S. V., Shah, S. K., Sheth, J. K., Sudeep, A. B. & other authors (2009). Systemic involvements and fatalities during chikungunya epidemic in India, 2006. *J Clin Virol* **46**, 145–149.

Tesh, R. B. (1982). Arthritides caused by mosquito-borne viruses. *Annu Rev Med* **33**, 31–40.

Van Bortel, W., Dorleans, F., Rosine, J., Blateau, A., Rousset, D., Matheus, S., Leparc-Goffart, I., Flusin, O., Prat, C. & other authors (2014). Chikungunya outbreak in the Caribbean region, December 2013 to March 2014, and the significance for Europe. *Euro Surveill* **19**.

Viennet, E., Knope, K., Faddy, H. M., Williams, C. R. & Harley, D. (2013). Assessing the threat of chikungunya virus emergence in Australia. *Commun Dis Intell Q Rep* **37**, E136–E143.